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利用綠豆左旋多巴增加種子鐵含量

Increasing iron content in seeds using mungbean-produced L-DOPA

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Increasing iron content in seeds using mungbean-produced L-DOPA

本論文係羅寶修君(R09623030)在國立臺灣大學農業化學系完成之碩士學位論文，於民國 111 年 8 月 16 日承下列考試委員審查通過及口試及格，特此證明

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List of Abbreviations

Abbreviation	Definition
GMOs	Genetically Modified Organisms
HPLC	high performance liquid chromatography
IDA	iron deficiency anemia
LC-MS/MS	liquid chromatography-tandem mass spectrometry
OB	organic bean
RBCs	red blood cells
WHO	World Health Organization

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摘要

長久以來，缺鐵影響著人類健康，因此充足之鐵營養相當重要，利用生物營養強化 (biofortification) 增加農作物中微量營養元素含量是解決此問題具有前景之方法。但是，有鑑於基因改造生物在環境與安全上之疑慮，使用基因工程實現之營養強化稻米並未被大眾完全接受；而相較於傳統育種速度緩慢且成功率低，生物刺激素 (biostimulant) 不受限於特定作物與品種之特性使其應用既快速且有通用性。本篇研究測試 L-DOPA 作為生物刺激素應用於鐵營養強化上，結果顯示在不影響阿拉伯芥種子產量之情況下，施用 L-DOPA 可以增加種子中之鐵含量。由於在田間施用外源 L-DOPA 不方便又昂貴，因此探討了以綠豆間作作為 L-DOPA 供應者之可能性，並透過定量種子中 L-DOPA 含量以鑑定出製造 L-DOPA 之綠豆品種。然而，當阿拉伯芥與綠豆間作時，阿拉伯芥之生長受到 L-DOPA 以外之化感物質 (allelochemicals) 抑制。根據這些結果判斷，若施用得當，L-DOPA 仍具有潛力作為鐵營養強化之生物刺激素；綠豆作為 L-DOPA 供應者之合適性則仍需要更進一步研究。綜合上述，本篇研究可為藉由生物刺激素實現生物營養強化之相關研究提供參考。

關鍵詞：左旋多巴 (L-DOPA)、高效液相層析儀 (HPLC) 、綠豆、貧血、鐵

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Abstract

Lack of iron (Fe) affects human health for a long time; therefore, appropriate Fe nutrition is important. To address the issue, biofortification that increases micronutrient contents in crops is a promising approach. Biofortification of rice achieved using genetic engineering is not well accepted by the public due to environmental and safety concerns over GMOs. Traditional breeding is slow and has only limited success. Biostimulants constitute a fast and versatile solution because they are not restricted to a particular crop or cultivar. In this study, L-DOPA was tested as a biostimulant for Fe biofortification and was shown to increase Fe content in Arabidopsis seeds without affecting the yield. Because the exogenous application of L-DOPA in the field is inconvenient and expensive, the use of mungbean as a L-DOPA donor by intercropping was investigated. Mungbean cultivars that produce L-DOPA were identified by quantifying L-DOPA in their seeds. However, Arabidopsis exhibited growth inhibition when intercropped with mungbean caused by allelochemicals other than L-DOPA. Based on these results, it is suggested that L-DOPA might be a potential biostimulant for Fe biofortification if applied properly, while further studies are needed to evaluate the suitability of mungbean as the donor. This study could be a reference for achieving biofortification through biostimulants in related studies.

Keywords: L-DOPA, HPLC, mungbean, anemia, Fe

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1. Introduction

1.1 Iron for plant

Iron (Fe) is one of the essential elements, needed in each growth stage of plants. As a cofactor, Fe is involved in a large number of biochemical reactions such as chlorophyll synthesis, photosynthesis, enzyme production, and respiration. Therefore, excess and shortage of Fe can lead to Fe toxicity and deficiency, respectively, which are detrimental to plant growth and development. Fe deficiency, which occurs mainly in high pH soil, is much more common in the environment compared with Fe toxicity, which usually occurs in waterlogged land. Although Fe is globally abundant in most soils, it is poorly bioavailable due to the low solubility, especially in alkaline soil. As a consequence, Fe deficiency is a major nutritional disorder of plants in many regions since over 30% of the cultivated land worldwide is calcareous. To cope with this limitation, plants have evolved two strategies for enhancing Fe uptake by their roots (Schmidt et al., 2020). Interveinal chlorosis is a typical symptom of Fe deficiency referring to the yellowing of young leaves tissues with veins that remain green (Jeong and Connolly, 2009). The yield and quality of plants will decrease as a result of the reduction of chlorophyll production. In severe cases, leaves will turn white and smaller and plants will be incapable of completing their life cycle. Hence, Fe deficiency is not only a problem with metal ions acquisition in plants

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but a noteworthy issue of enormous economic importance in the world.

1.2 Iron for human beings

Fe is the most abundant trace element in the human body and contributes to vital functions. They could be roughly covered by three aspects, oxygen transportation from lungs to tissues, electron transport chain within cells, and a critical cofactor of enzyme systems. People intake of Fe from the diet is classified into two forms: heme iron and non-heme iron. Heme iron, only derived from hemoglobin and myoglobin in foods of animal origin, is easily absorbed by the human intestine (15-35%). In comparison, non-heme iron is found in plant-based foods and animal tissues and is less bioavailable (2-20%) because it is susceptible to the effects of other components in food, such as phytate, calcium, and polyphenols (Hurrell and Egli, 2010). Despite its low bioavailability, non-heme iron generally remains the primary contributor to iron nutrition because its abundance in the diet is many times that of heme iron (Monsen et al., 1978).

Human requirements of Fe vary depending on the age and sex (Supplementary table S1). The little content of Fe contained in breast milk is enough for early infancy. The need for Fe increases markedly with the growth of body mass between ages 1 to 6 and during adolescence but changes little in adults. Gender difference in Fe requirement emerges

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after menarche in females since lots of Fe is lost in menstrual blood (WHO, 2004). In order to balance the Fe homeostasis in human bodies, the loss of Fe through bleeding, sweating, and excretion needs to be met by intake as well as the increased demand in certain growth stages. Otherwise, it would lead to Fe deficiency which is usually accompanied by anemia.

Anemia is a condition in which the number of red blood cells or the hemoglobin concentration within them is lower than normal resulting in several symptoms including fatigue, irregular heartbeats, shortness of breath, and headaches. Various reasons can cause anemia not only Fe deficiency but also nutritional deficiencies in vitamins A and B12; hemoglobinopathies; and infectious diseases. The mechanism of Fe deficiency leading to anemia is that Fe binds with oxygen in the heme structure, which is a component of hemoglobin contained in red blood cells (RBCs). Hence, without enough Fe, RBCs will not be produced at normal levels (Hurrell, 1997).

Anemia constitutes the most common nutritional disorder in humans, affecting cognitive development, immune system, and work productivity. According to a survey by the WHO, two billion people worldwide suffer from anemia, especially young children and pregnant women, and in particular in the poorest regions of the world (Supplementary Figure S1). Among these 2 billion, Fe deficiency approximately accounted for 50% (De Benoist et al., 2008). To solve this problem, biofortification of iron in crops is a promising

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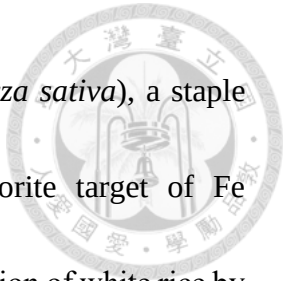

approach since plants are the major source of dietary Fe. Rice (*Oryza sativa*), a staple food for more than half of the world's population, is the favorite target of Fe biofortification. The WHO estimated that increasing the Fe concentration of white rice by 10-fold would be the most effective strategy to combat IDA. Although this goal was already achieved by the Golden Rice project, the use of this rice to improve IDA has not been achieved because of concerns from the public over the transgenic methods that were used to produce the Golden Rice.

1.3 L-DOPA might be a biostimulant of Fe accumulation

L-3,4-dihydroxyphenylalanine (L-DOPA), a non-proteinogenic amino acid produced by the hydroxylation of tyrosine, is the precursor of various biologically active compounds such as the catecholamine neurotransmitters dopamine and adrenaline, or the ubiquitous pigment melanin (Supplementary Figure S2). Melanin is produced from dopaquinone, which is generated by the oxidation of L-DOPA. In addition, dopamine can be produced via L-DOPA decarboxylation and further form noradrenaline and adrenaline through hydroxylation and methylation successively, all of which are important neurotransmitters (Kong et al., 1998; Kulma and Szopa, 2007; Steiner et al., 1996). Due to the characteristic that L-DOPA crosses the blood-brain barrier, which dopamine cannot,

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it can be supplied exogenously to boost dopamine levels in the brain. Therefore, L-DOPA is used in the treatment of Parkinson's disease (Birkmayer and Hornykiewicz, 1961), a brain disorder caused by the severe depletion of dopamine that mainly affects the motor system. After the introduction of the high-dose oral L-DOPA regimen achieved great success in 1967, the vital status of L-DOPA as a therapeutic agent of Parkinson's disease was established. Even though new molecules were added to the therapy one after another along with the progressing study, L-DOPA is still irreplaceable in the treatment of Parkinson's disease.

The attention to L-DOPA has never diminished since its discovery; however, the information on its action mode in plants remains far from being fully understood. So far, L-DOPA is known for serving as an allelochemical in several legume species, especially at high levels in fava bean (*Vicia faba*) and velvet bean (*Mucuna pruriens*) (Guggenheim., 1913; Nishihara et al., 2005). Allelochemicals are molecules that plants release for adapting to adverse environments by affecting the growth and development of neighboring plants. Previous studies have shown that L-DOPA has herbicide-like properties. The growth of lettuce was suppressed proportionally to the increasing concentration of L-DOPA (Nishihara et al., 2005). L-DOPA inhibits the growth of certain plants at concentrations ranging from 0.5 to 2 mM (Supplementary Figure S3; Soares et al., 2011). Due to the high content of L-DOPA, velvet bean has abilities such as

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suppression of weeds and tolerance to pests (Fujii et al., 1991). Regarding the physiological functions and toxicity mechanisms of L-DOPA in plants, the current consensus is that the reaction of the catecholamine group with Fe can produce free radicals, thereby inhibiting key enzymes and causing cell damage (Hašková et al., 2011; Venarucci et al., 1999). Interestingly, a microarray analysis has demonstrated that L-DOPA treatment increased the expression of Fe uptake genes in Arabidopsis, indicating an association with the regulation of metal homeostasis (Golisz et al., 2011), simultaneously, offering the possibility to use L-DOPA as a biostimulant, which was defined as the substance that improves nutritional efficiency, abiotic stress tolerance and/or crop quality traits of plants, regardless of its nutrient content in du Jardin (2015). While the disclosure is relevant to elucidate the mode of action of L-DOPA, it could also be leveraged for stimulating the uptake of Fe for biofortification purposes.

1.4 Mungbean (*Vigna radiata*)

Originating from India, mungbean is widely grown in tropical and subtropical regions, including India, China, Vietnam, Malaysia, Thailand, and Taiwan as a short-term and warm-season legume crop (Mohan Naik et al., 2020). It is generally below 125 cm tall and highly branched having trifoliate leaves and 10-25 flowers per pedicel that turn

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into cylindrical pods via self-pollinating. The morphology varies from erect, semi-erect to prostrate (Lambrides and Godwin, 2007). The nutritional content of mungbean consists of 51% carbohydrates, 24-26% protein, 4% minerals, and 3% vitamins (Afzal et al., 2008). As a food, mungbean has been popular in Asia since ancient times. It can be consumed as seeds, sprouts, or an ingredient in drinks, desserts, and soups. For people in developing countries, mungbeans play an important role in protein sources. In farming, mungbean is often cultivated as a preceding crop before cereal crops or as an intercrop to increase the harvest because the ability of nitrogen fixation from mungbean roots can fertilize the soil (Zheng et al., 1997). In many areas, mungbeans are also planted after natural disasters to compensate for crop losses by taking advantage of their drought tolerance and short lifecycle. In summary, mungbean can be used as green manure, fodder, and human food, and has considerable value in agricultural function and economy.

1.5 Scope of the thesis

In this study, the potential use of L-DOPA as a biostimulant to achieve biofortification was evaluated. L-DOPA was shown to induce the expression of Fe uptake genes in Arabidopsis roots, however, it is also known for its inhibitory effect on growth. It was therefore necessary to check if L-DOPA could be used to boost Fe uptake without

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compromising the yield.

The exogenous application of L-DOPA in the field would be costly and laborious, which would make this strategy unsustainable in populations with limited resources who are the ones suffering most from iron deficiency-induced anemia. Many legumes are known to secrete L-DOPA through their roots, and could potentially be used as a source of L-DOPA in the field. The well-known L-DOPA-producing species are fava bean and velvet bean; however, these species are not widely cultivated in Asia. Although mungbean has not been proven to produce L-DOPA at high concentrations, we hypothesized that some cultivars might. In the present study, we took advantage of the availability of a large collection of mungbean genetic resources at the World Vegetable Center and from Prof. Cheng-Ruei Lee (Institute of Plant Biology, National Taiwan University) to screen for mungbean accessions containing L-DOPA. L-DOPA is secreted by roots but also accumulates in the seeds, and the screening could therefore be carried out using dry seeds.

Overall, this study includes three primary aims. First, to determine whether the application of L-DOPA will increase Fe uptake in Arabidopsis. Second, to screen high L-DOPA content cultivars out of mungbean seeds. Last, to examine the changes of Fe content in Arabidopsis which was intercropped with mungbean in a pot. The results would indicate if L-DOPA can be applied for Fe biofortification and if mungbean is a suitable plant as a donor of L-DOPA.

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2. Materials and Methods

2.1 Plant materials and chemicals

Seeds of 50 cultivars of mungbean seeds were purchased from the World Vegetable Center (Supplementary table S2), bags of organic mungbean, adzuki bean (*Vigna angularis*), and soybean (*Glycine max*) were purchased from a supermarket, and bags of *Sesbania cannabina* and sun hemp (*Crotalaria juncea*) were purchased online. The accession of Arabidopsis plants used in this study is the Columbia-0. Methanol (Echo chemical, CAS-No: 67-56-1) and phosphoric acid (Nippon Shinyaku, CAS-No: 7664-38-2) used for the preparation of the mobile phase on HPLC (Hitachi High-Technologies Corporation, Tokyo, Japan) are of analytical grade.

2.2 Preparation of materials for growing plants

2.2.1 Soil and pots

The soil for growing Arabidopsis and mungbean was mixed homogeneously with one part of perlite, one part of vermiculite, and four parts of potting soil (Jiffy Group, Netherlands) that contains nutrients and organic compounds. Pots were filled with the soil pressed compactly and the excess soil was removed to flatten the surface.

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2.2.2 Preparation of hydroponics

The hydroponic system consisted of a box (12 × 9.5 cm) with a transparent lid, a white paper board, caps of microcentrifuge tubes, and nutrient solution (ES media) (Estelle & Somerville, 1987). The paper board was trimmed to match the box size and poked with twelve holes for holding caps of microcentrifuge tubes. A small hole was made in the middle of each cap and filled with 0.7% agar (0.7g of agar in 100 mL of ES media). When the agar had solidified, Arabidopsis seeds were placed on it. The nutrient solution needed to be changed twice a week. Supplementary table S3 shows the formula of the nutrient solution.

2.3 Growth conditions

2.3.1 L-DOPA treatments

Arabidopsis plants were grown in pots treated with 100 mL of various concentrations of L-DOPA (0, 10, 20, 50, 100, 200 ppm) once a week. After two months, these seeds were harvested for iron quantification. These Arabidopsis plants were grown in a growth chamber under 16 h light / 8 h dark at 22 °C.

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2.3.2 Mungbean serving as a source of L-DOPA

A high L-DOPA mungbean cultivar and a low one were planted. To germinate nearly simultaneously, these mungbeans were first soaked in ultrapure water for ten hours. After the mungbeans had grown for three weeks, Arabidopsis plants were grown together with them in pots. Iron quantification of the Arabidopsis was conducted one month later. Nine pots were placed in a tray irrigated with 1 L ultrapure water from the bottom once a week. No fertilizer was applied in this experiment. All plants used were grown in a growth chamber under 16 h light / 8 h dark at 22 °C.

2.3.3 Confirmation of mungbean metabolites inhibiting Arabidopsis growth

Arabidopsis plants were grown in hydroponics for three weeks. Afterward, the shoots of Arabidopsis were harvested and weighed the fresh weight. In the last two weeks, the nutrient solution of treatment groups was added with the extract from the soil used for growing mungbean to reach the concentration of 0.25% (1 mL soil extract in the 399 mL nutrient solution). The extract method is mentioned in chapter 2.5. These Arabidopsis plants were grown in a growth chamber under 16 h light / 8 h dark at 22 °C.

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2.4 Iron quantification

The standard curve was prepared with a range of Fe concentration: 0, 1.25, 2.5, 5, 10, 20, and 40 μg of Fe in glass test tubes, corresponding to 0, 0.45, 0.9, 1.79, 3.58, 7.17, and 14.34 μL of a solution of Fe-EDTA at 50 mM concentration dissolved in water. Samples (Arabidopsis seeds) were dried for 12 hours in an oven at 60 °C and transferred 8-15 mg into the glass test tube. The standards and the samples prepared were treated with the same following steps. 225 μL of 65% (v/v) nitric acid (HNO_3) was pipetted onto the samples at the bottom of the tubes, which should be held straight, without pipetting any drop on the tube wall. Gently tapping on the bottom of the tube could ensure that the whole sample is soaked in acid. The tubes were placed into a heat block and heated at 96 °C until the samples were dissolved (up to 6 hours) and then taken out of the heat block and left on a rack in the fume hood. After 150 μL of 30% (v/v) hydrogen peroxide (H_2O_2) was added, the tubes were placed back on the heat block at 56 °C. When the samples were completely discolored (up to 2 hours), 225 μL of ultrapure water was added to each tube. The tubes can be stored overnight at 4 °C and measured the day after if needed. BPDS solution, which cannot be stored for later use, containing 1 mM BPDS, 0.6 M sodium acetate, and 0.48 M hydroxylamine dissolved in ultrapure water was prepared. 15 μL of the sample was mixed with 235 μL of BPDS solution and measured absorbance at 535 nm using a spectrophotometer. The absorbance values were converted to concentrations

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in μg against the standard curve equation. The Fe concentrations in $\mu g.g^{-1}$ of the samples were calculated by dividing by the sample dry weights. The method was described in Pan et al. (2015).

2.5 Soil extraction

The whole pot of soil (about 60 g) was taken to a flask and added with 400 mL of 80% methanol or ultrapure water. An ultrasonicator was used for mixing the extract solution for 20 minutes. After that, the extract solution was filtered with a 90 mm filter first and then centrifuged at 3000 rpm for 5 min at room temperature. The supernatant was taken out for 300 mL and dried with a rotary evaporator, then 6 mL of 80% methanol was used for resuspension to concentrate 50 times. In the end, the concentrated extract solution was collected and filtered with a 0.45 μm filter.

2.6 Sample extraction

Mungbean seeds were ground into powder and 100 mg were weighed and placed into microtubes with of ice-cold extraction solution of 1500 μL of HCl. After mixing the sample for 10 min and centrifuging at 15000 rpm for 10-15 min at 4 °C, the supernatant was transferred to a new tube. After three rounds of centrifugation and supernatant

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transfer, the samples were analyzed with HPLC (Siddhuraju and Becker, 2001).

2.7 Analytical conditions

2.7.1 High-performance liquid chromatography (HPLC)

The detection of L-DOPA was conducted on a Hitachi HPLC D-2000 system which is composed of a L-2455 diode array detector, a L-2200 autosampler, and a L-2130 pump. A reverse phase C_{18} column, Cosmosil 5C_{18}-AR-II (4.6 × 250 mm, 5 μm, Waters, Milford, MA, USA), was used at 30 °C. The mobile phase consisted of solvent A (pH 2.0 H_2O adjusted with phosphoric acid) and solvent B (70% methanol). The injection volume was 10 μL and the detection wavelength was monitored at 282 nm. The settings of gradient and flow rate were shown in Supplementary table S4. The HPLC was kindly lent by Prof. Pei-Jen Chen (Department of Agricultural Chemistry, National Taiwan University)

2.7.2 Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

L-DOPA in mungbeans was confirmed using an Acquity UPLC (Waters, Milford, MA, USA) coupled with a timsTOF Pro mass spectrometer (Bruker Daltonics, Billerica,

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MA, USA). The chromatography was performed on a BEH amide column (2.1 × 150 mm, 1.7μm) at 40 °C with a mobile phase consisting of solvent A (10 mM ammonium formate in H_2O + 0.1% formic acid) and solvent B (10 mM ammonium formate in 95% ACN/5% H_2O + 0.1% formic acid). The injection volume was 5 μL. The settings of gradient, flow rate, and mass spectrometry were shown in Supplementary table S5 and Supplementary table S6. The analysis was conducted in collaboration with Bruker. Similar results were obtained using the LC-MS/MS system from the Metabolomics core facility of Academia Sinica.

2.8 Statistical analysis

The significance of the difference in the mean of two treatments was determined by the one-tailed Student's t-test (P<0.05) using Microsoft Excel.

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3. Results

3.1 L-DOPA increases the Fe concentration in Arabidopsis seeds

The L-DOPA treatment has been reported to increase the expression of Fe uptake genes in Arabidopsis in a previous study (Golisz et al., 2011), it was assumed that L-DOPA could increase the Fe concentration in plants. To confirm this hypothesis, Arabidopsis plants were treated with exogenous L-DOPA in pots. Under the 6 different treatments with L-DOPA concentrations of 0, 10, 20, 50, 100, and 200 ppm, the seed yield of Arabidopsis was not affected (Figure 3.1), suggesting that these concentrations are insufficient to exhibit its herbicide-like property. Then, these seeds were digested with HNO_3 for quantifying Fe to investigate whether the Fe content was increased. The result showed that the Arabidopsis watered with L-DOPA had 33% to 64% higher Fe content in the seeds than the control group (Figure 3.2). Based on these results, L-DOPA was confirmed to increase the Fe concentration in the seeds without affecting the yield, which implies that it could be used as a biostimulant for Fe biofortification.

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3.2 L-DOPA in mungbean seeds was detected by HPLC and LC-MS/MS

Since the HPLC technology has been long-term developed and widely applied for the analysis of various compounds in different fields, it was used in this study to qualify L-DOPA in mungbean seeds. After analysis, the chromatogram showed that the standard and mungbean samples all had consistent peaks at the retention time of 0.84 min (Figure 3.3). To examine whether the molecule of the monitored peak was L-DOPA, the samples were sent to mass spectrometry (MS). The mass spectrogram detected a peak of 198.0762 m/z which corresponded to the mass of [M+H] of L-DOPA. To further confirm, the molecule was fragmented and passed to MS/MS. Two peaks of 139.0391 and 152.0708 m/z were revealed which corresponded to the daughter ions of L-DOPA demonstrating that the molecule detected was L-DOPA (Figure 3.4). In short, HPLC was verified by LC-MS/MS that it can detect L-DOPA, so the following analysis would be conducted with this instrument.

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3.3 All tested 50 accessions of mungbean seeds contain L-DOPA

Since several legume species have been proven to contain L-DOPA in their seeds (Ramya and Thaakur, 2007), mungbean has the potential as well. To fast examine if mungbean produces L-DOPA, 50 accessions of mungbean seeds, whose genome sequences have been constructed and constituted a part of the core collection (Schafleitner et al., 2015), were analyzed by HPLC. After screening, all of them are found to contain L-DOPA (Figure 3.5) though generally at a low concentration of 20 $\mu g.g^{-1}$. VI000099AG had the highest L-DOPA concentration at about 61 $\mu g.g^{-1}$. This result suggests that it might be possible to use mungbean as a L-DOPA donor. Among them, the highest L-DOPA-producing cultivar (VI000099AG) and the lower ones (VI003135B-BL and VI004973B-BLM) were selected for the following experiments.

To discover the plants that have higher L-DOPA content, other legumes commonly found in Taiwan were also analyzed, such as adzuki bean, soybean, sun hemp, and *Sesbania cannabina*. Figure 3.6 depicts that most of these tested legumes only contained L-DOPA levels below 10 ppm, similar to that in most of the mungbean cultivars tested. In view of these results, mungbean is the most suitable one among the tested legumes.

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3.4 Mungbean inhibits Arabidopsis growth

To investigate whether mungbean can serve as the source of L-DOPA to increase Fe content in Arabidopsis, the two plants were co-cultivated in the same pot. The result showed these Arabidopsis plants, regardless of the cultivar they are grown with, are much smaller in size and weight with lower Fe content compared with the control (Figure 3.7, Figure 3.8, Figure 3.9, Figure 3.10). In this outcome, there is no difference between the Arabidopsis grown with the different two mungbean cultivars, which means the mungbeans likely secreted other metabolites that inhibited the growth of Arabidopsis rather than L-DOPA. In conclusion, mungbean might not be an appropriate L-DOPA source for Arabidopsis in the intercropping system because of its strong allelopathic properties caused by unidentified molecules. It remains possible that mungbean could be used with crop species that are more tolerant than Arabidopsis to the toxicity of these molecules.

3.5 Mungbean secretes phytotoxic metabolites

In the metabolite profile of mungbean seeds by mass spectrometry, some phytotoxic compounds were found, such as Acremin F and m-Fluoro-DL-phenylalanine, but it is uncertain if they were related to the growth inhibition. To validate that the growth

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inhibition of Arabidopsis is caused by the metabolites secreted by mungbean and not nutrient depletion or competition, metabolites were extracted from the potting soil used to grow mungbean and supplied exogenously in the nutrient solution of Arabidopsis plants cultivated in hydroponics. In this case, the nutrient would be sufficient by changing the nutrient solution twice a week, and the most likely factor that affects Arabidopsis would be the soil extracts containing the metabolites of mungbean. On the first try, the metabolites extracted with ultrapure water did reduce the rosette weight in Arabidopsis plants, yet the difference was much less pronounced than what was observed in soil-grown plants (Figure 3.11), possibly due to an inefficient extraction because of adsorption of the metabolites to the soil. In order to extract as many metabolites as possible, the extraction reagent was changed to 80% methanol. However, this resulted in a weak growth of the plants subjected to the mock treatment, which grew worse than the plants subjected to the actual extract (Figure 3.12, Figure 3.13). It was concluded that the 0.25% methanol in the nutrient solution was the main reason for the growth inhibition. Plant roots are so sensitive to methanol that brief exposure to 10% methanol could inhibit root growth (Hemming et al, 1995). Long-term exposure to methanol is even more harmful. Merely 1% methanol resulted in a 45% reduction in onion root growth (Fiskesjo and Levan, 1993). On the other hand, plants in the treatment group grew a bit better than the mock might suggest that certain components in the soil extracts mitigate the toxicity of

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methanol. For making the phytotoxic activity of mungbean metabolites manifest clearly, the methanol concentration in the nutrient solution should be lower.

3.6 Variability of allelopathy from mungbean

In the intercropping system, the intercrop should not bring negative effects on the target crop. Hoping to find less toxic accessions, the extent of allelopathic effects among mungbean accessions was investigated by cultivating Arabidopsis plants in the pots that were used to grow mungbean accessions. Although Figure 3.14 depicts that Arabidopsis plants were all strongly inhibited besides the control, there are some variabilities in the inhibition effect among different accessions illustrated in Figure 3.15. In addition, all these pots were irrigated from the bottom, which means the allelochemicals secreted by mungbean roots may move through the water. However, the control plants were not affected by the inhibition effect, suggesting the allelochemicals were binding in the soil.

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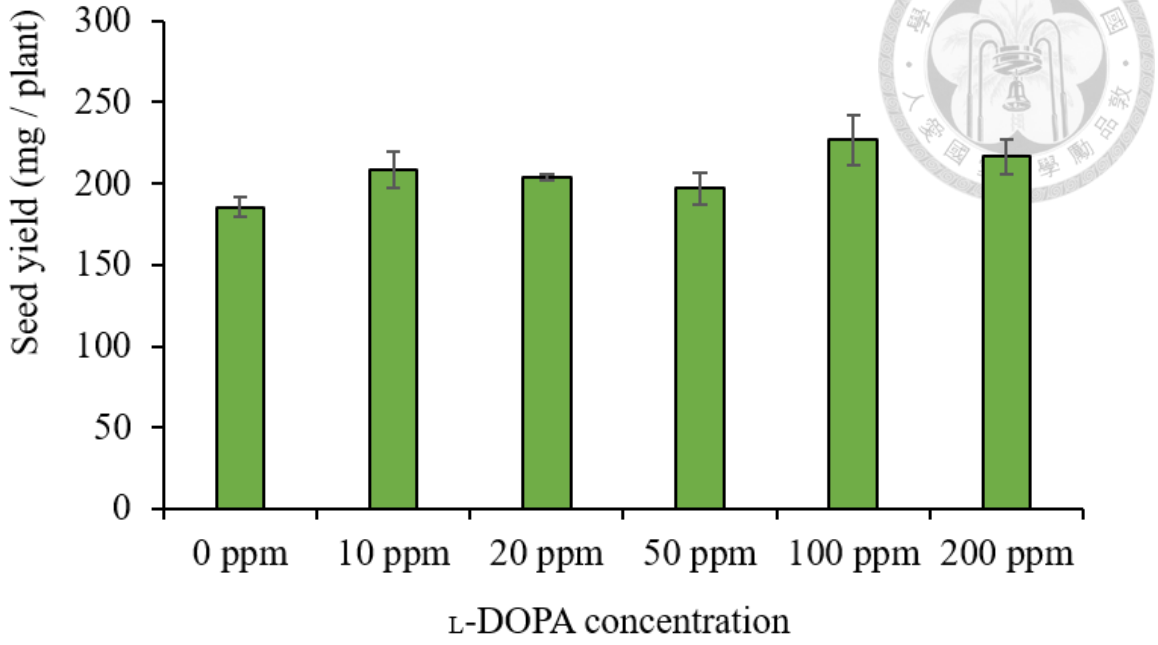

Figure 3.1 Seed yield of Arabidopsis irrigated with different concentrations of L-DOPA

Measurement of seed yield in wild-type Arabidopsis treated with 0, 10, 20, 50, 100, and 200 ppm L-DOPA. L-DOPA does not affect the seed yield of wild-type at all the concentrations tested. Results are means of six plants each, error bars correspond to standard deviation (n=6). No significant differences comparing each concentration to 0 ppm were found using Student's t-test.

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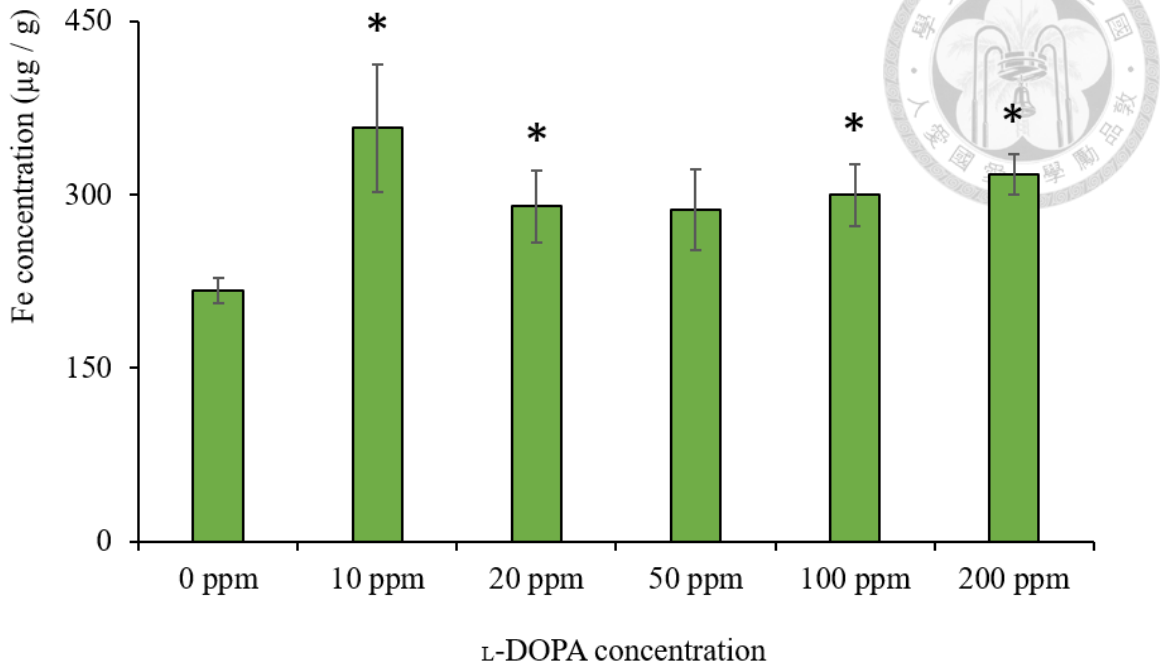

Figure 3.2 Fe content in Arabidopsis seeds under different concentrations of L-DOPA

Quantification of Fe content in wild-type Arabidopsis treated with 0, 10, 20, 50, 100, and 200 ppm L-DOPA. The induction of Fe uptake was achieved at most of the concentrations of L-DOPA treatments. Results are means of six plants each, error bars correspond to standard deviation. There were significant differences found under 10, 20, 100, and 200 ppm L-DOPA treatments using one-tailed Student's t-test. Asterisks represent a significant difference (*P<0.05).

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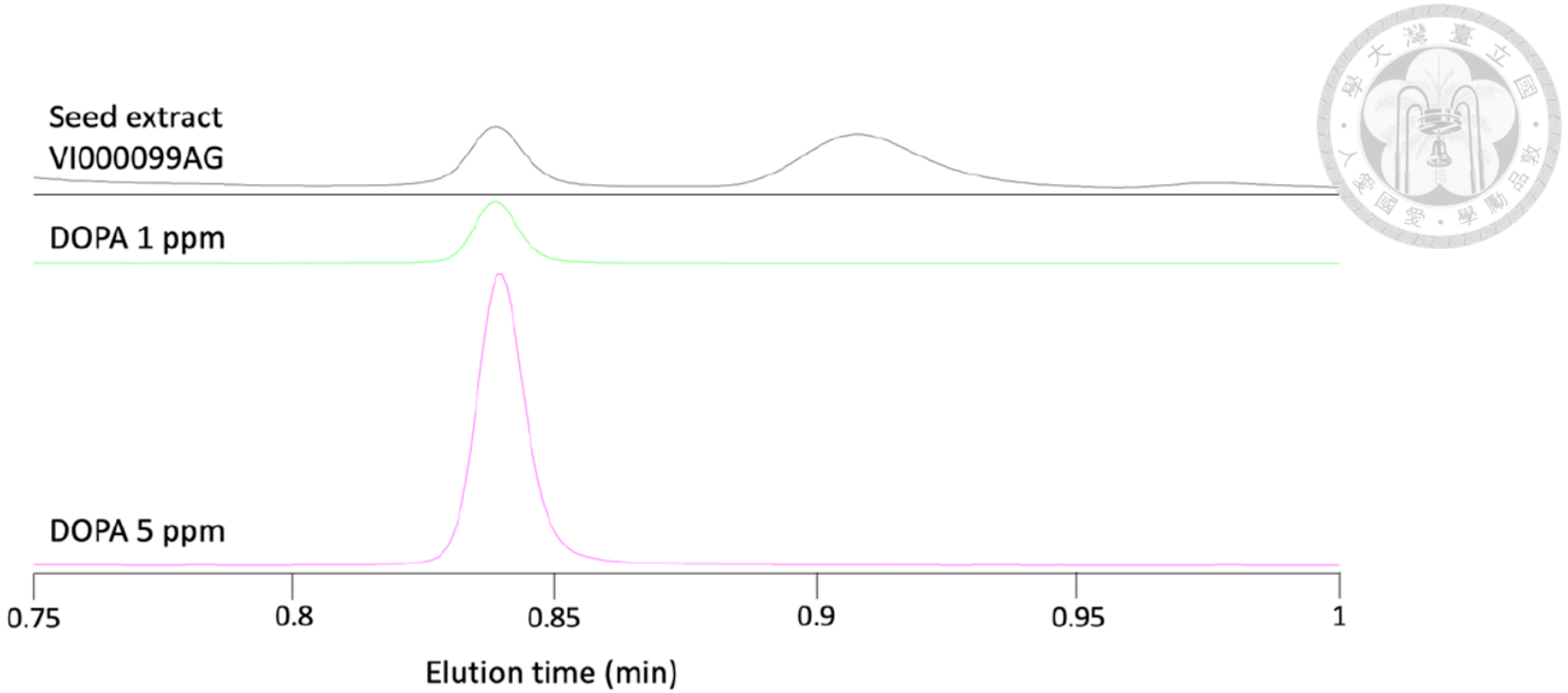

Figure 3.3 Confirmation and relative quantification of L-DOPA in the VI000099AG cultivar

L-DOPA in mungbean seed was detected using UPLC. The chromatogram illustrated the mungbean cultivar (VI000099AG) and L-DOPA standards with 1 and 5 ppm all had peaks at the retention time of 0.84 min

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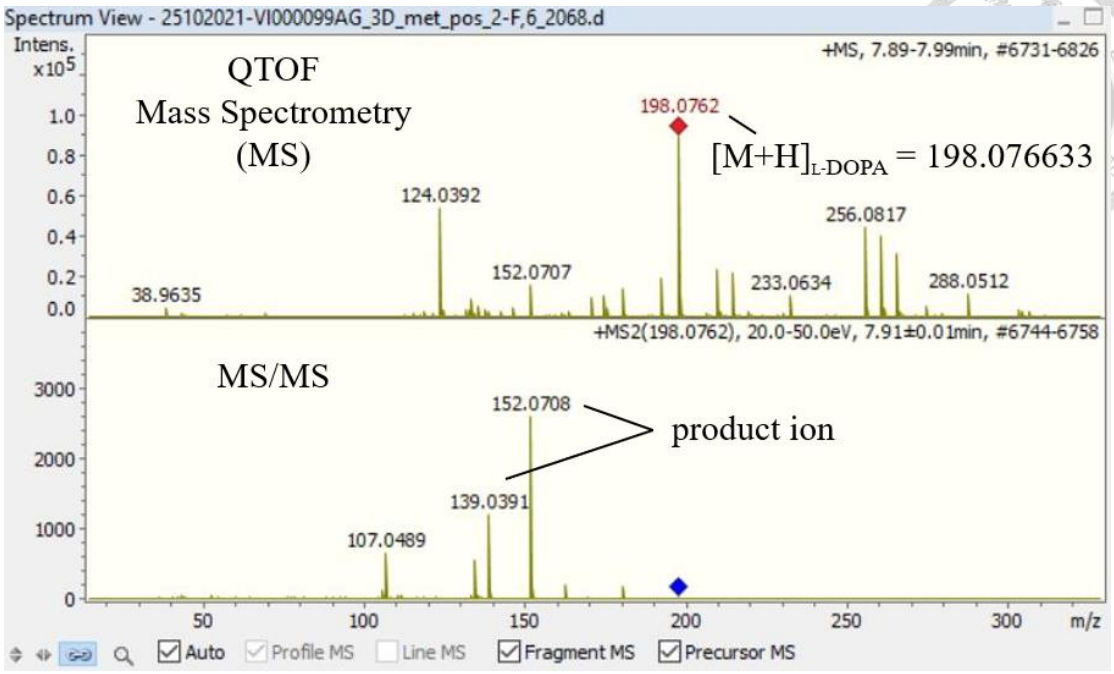

Figure 3.4 MS (upper) and MS/MS (lower) spectrum of L-DOPA

Analysis of mungbean cultivar VI000099AG by LC-MS/MS. A peak of 198.0762 m/z corresponded to the mass of [M+H] of L-DOPA was detected in the MS spectrum; two peaks of 139.0391 and 152.0708 m/z corresponded to the product ions of L-DOPA were detected in the MS/MS spectrum.

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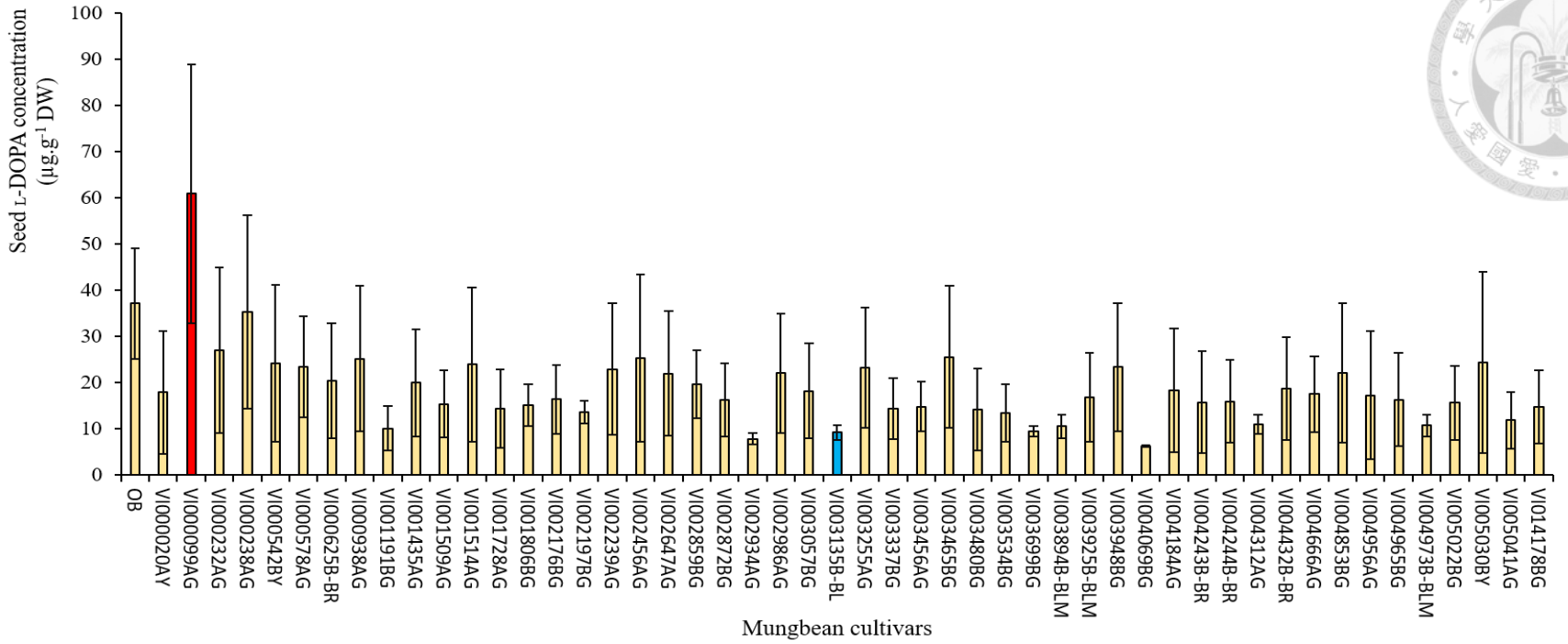

Figure 3.5 L-DOPA screening of 50 cultivars of mungbean seeds

Relative quantification of L-DOPA in 50 cultivars of mungbean seeds by chromatography. The result of the screening showed all of them contains L-DOPA. The L-DOPA concentration in the VI000099AG (61 μg.g^{-1}) was higher than the others. Results from UPLC and HPLC with a similar trend are combined into one graph, and error bars correspond to the two data. OB, organic bean. Red bar, the highest L-DOPA concentration cultivar.

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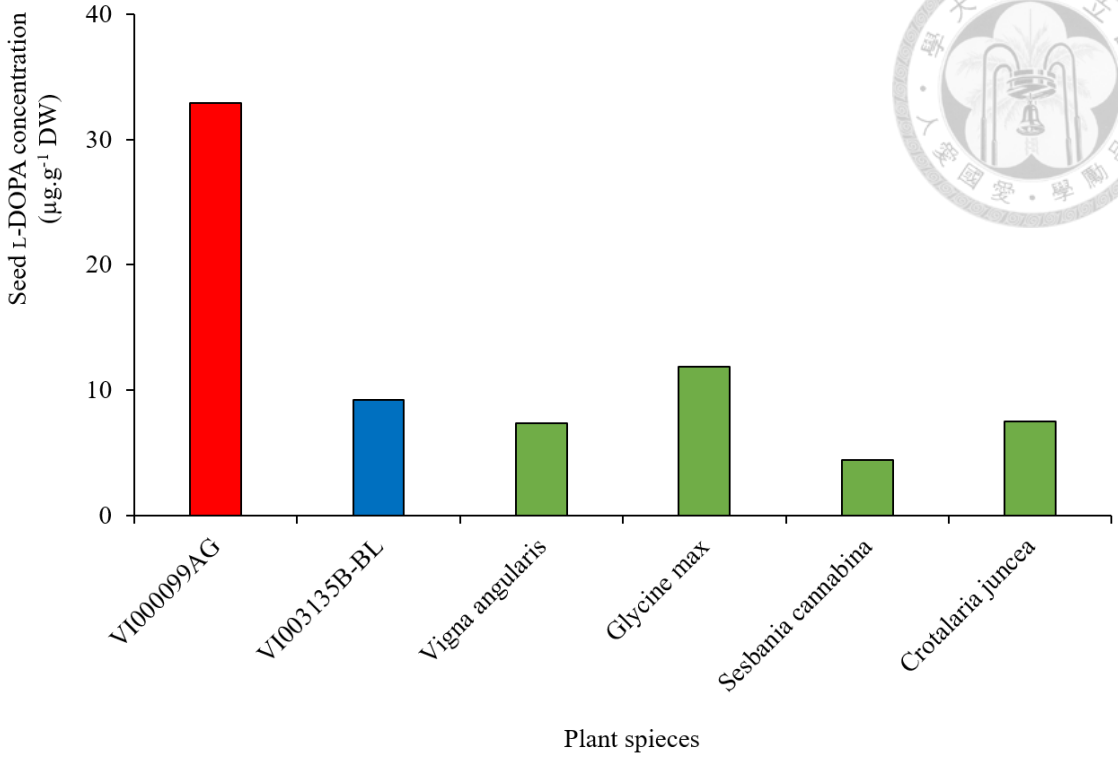

Figure 3.6 L-DOPA screening of the legumes

Relative quantification of L-DOPA in various legumes (mungbean, *Vigna angularis*, *Glycine max*, *Sesbania cannabina*, *Crotalaria juncea*) on HPLC. Only VI000099AG had higher levels of L-DOPA (32.9 μg.g^{-1}), while the other tested legumes had levels approximately as low as the VI003135B-BL, which is the low L-DOPA-producing mungbean cultivar.

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Figure 3.7 Growth inhibition of Arabidopsis grown with mungbean

Representative picture of the three-week-old wild-type soil-grown Arabidopsis plants co-cultivated with different mungbean cultivars (VI000099AG, VI004973B-BLM, and VI003135B-BL) used for the measurement of rosette fresh weight. Scale bar, 1 cm.

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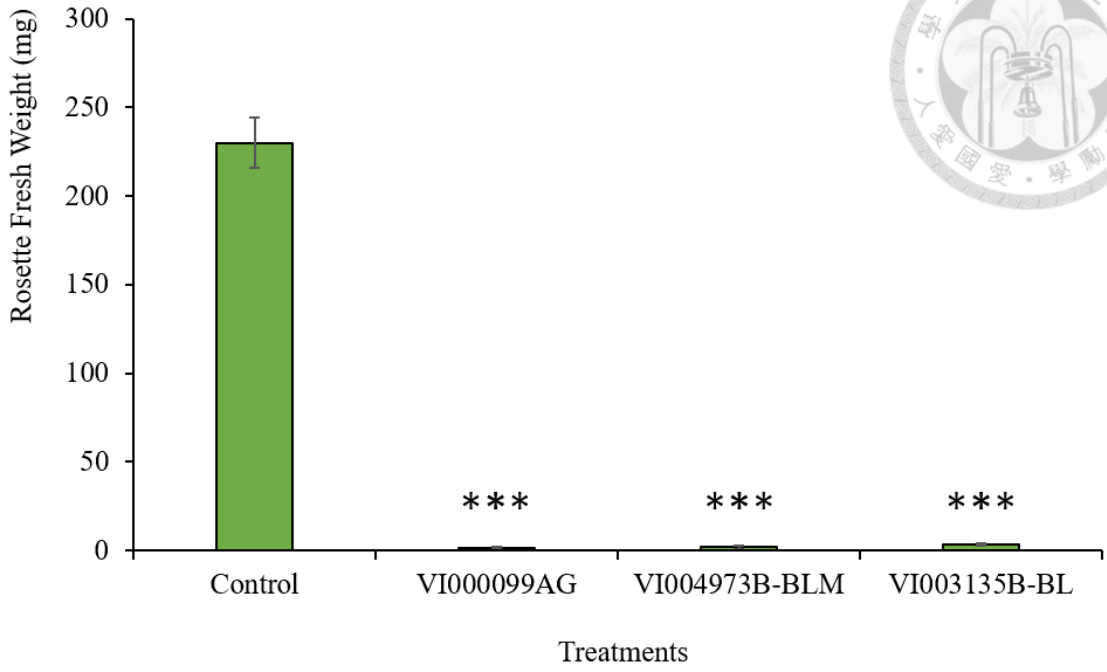

Figure 3.8 Soil-grown Arabidopsis rosette fresh weight

Analysis of rosette fresh weight in three-week-old wild-type soil-grown Arabidopsis plants co-cultivated with different mungbean cultivars (VI000099AG, VI004973B-BLM, and VI003135B-BL). The rosettes co-cultivated with different mungbean cultivars grew significantly smaller than they grew alone. Two independent biological repeats, carried out with three to eight plants each, were performed with similar results. Control was Arabidopsis plants grown alone. Statistical significance has been estimated as compared with control using one-tailed Student's t-test. Asterisks represent a significant difference (***P<0.001).

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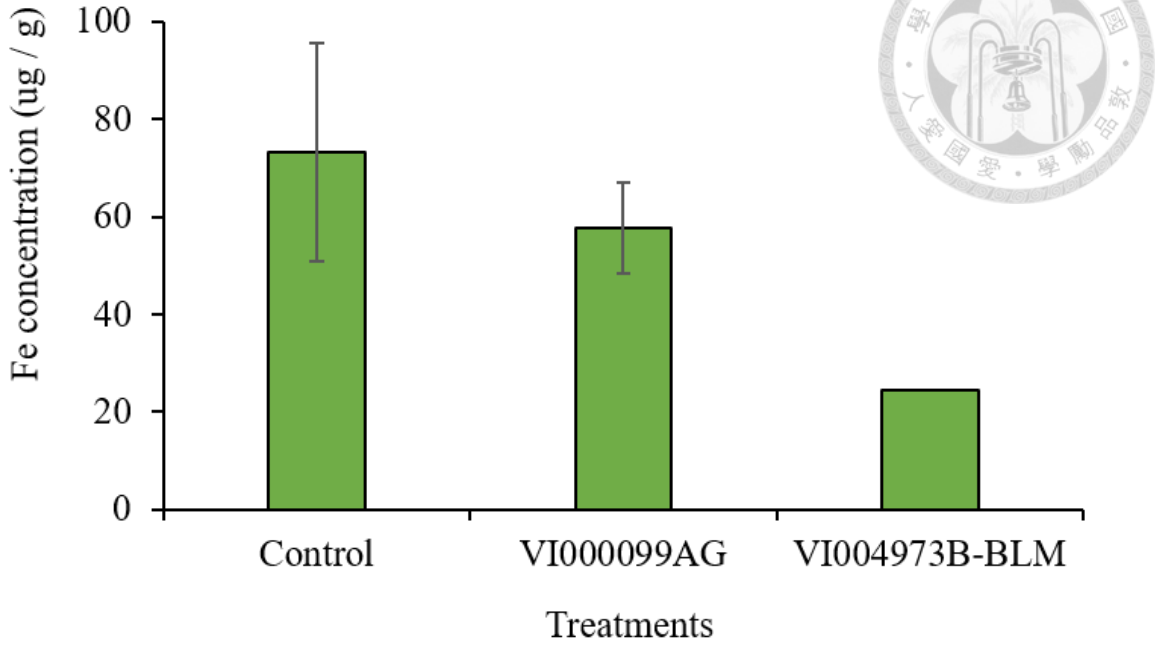

Figure 3.9 Fe content in soil-grown Arabidopsis rosette

Analysis of Fe content in three-week-old wild-type soil-grown Arabidopsis plants co-cultivated with different mungbean cultivars (VI000099AG, and VI004973B-BLM). The Fe content was lower in rosettes co-cultivated with different mungbean cultivars than rosettes grown alone. Results are means of two replicates carried out with three plants each, error bars correspond to standard deviation. Control was Arabidopsis plants grown alone.

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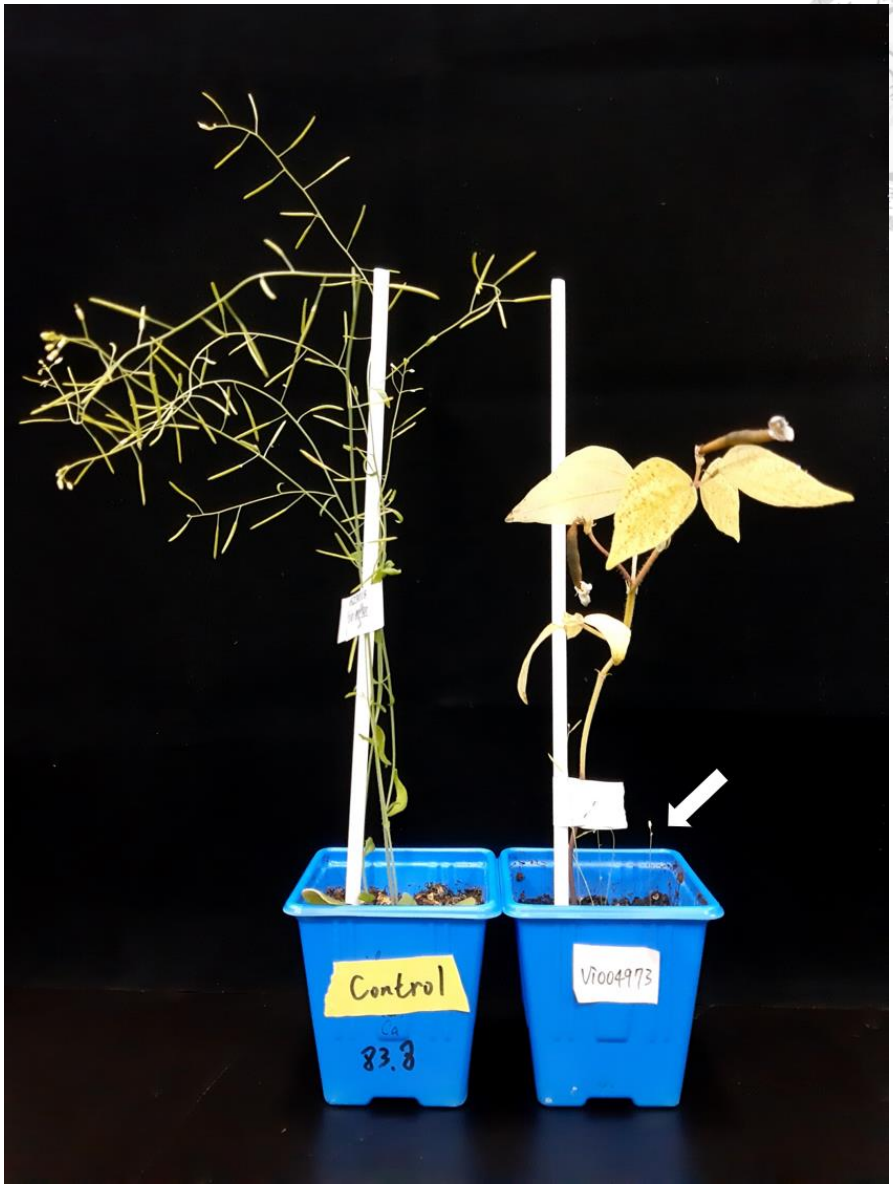

Figure 3.10 Comparison of size and growth stage of Arabidopsis

Representative picture of the six-week-old wild-type soil-grown Arabidopsis plants co-cultivated with mungbean (VI004973B-BLM). The two pots of Arabidopsis were significantly different in size, but at roughly the same growth stage. Arrow indicates the inhibited Arabidopsis plants.

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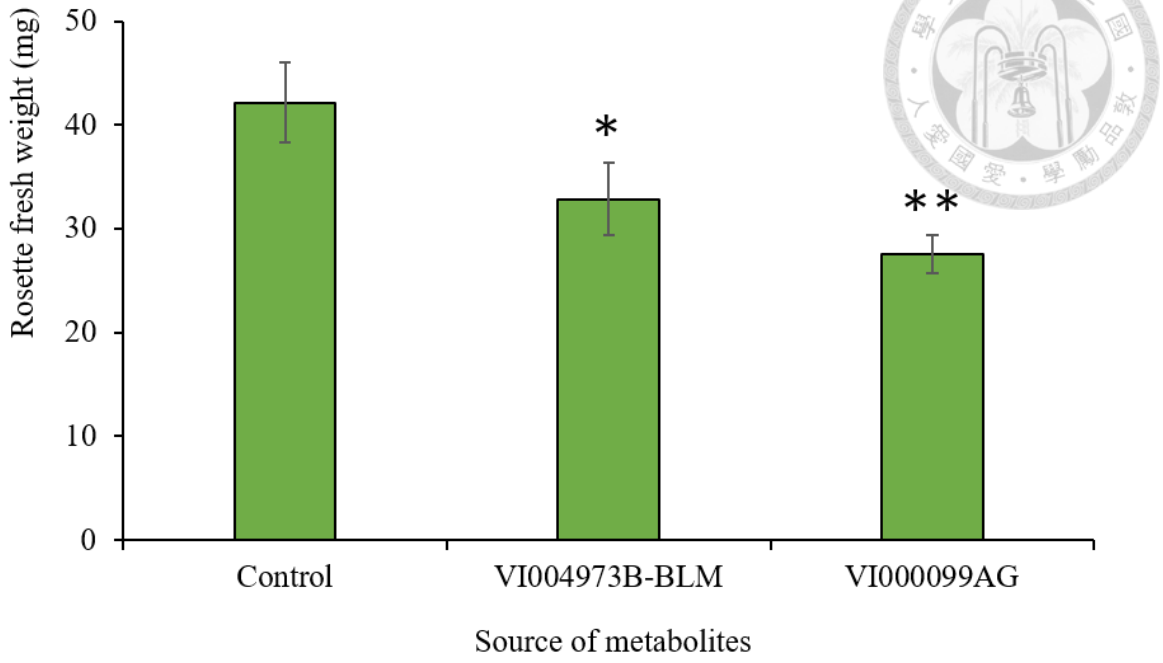

Figure 3.11 Hydroponic Arabidopsis rosette fresh weight subjected to water-extracted mungbean metabolites

Analysis of rosette fresh weight in three-week-old wild-type hydroponic Arabidopsis plants subjected to metabolites from different mungbean cultivars (VI000099AG, VI004973B-BLM) that were extracted with ultrapure water. The rosettes that grew in the nutrient solution containing metabolites from mungbean were lighter than in the nutrient solution with no additives. No biological repeat was carried out. Each treatment has 11-12 plants. Control was Arabidopsis plants grown in the nutrient solution without mungbean metabolites. Statistical significance has been estimated as compared with control using one-tailed Student's t-test. Asterisks represent a significant difference (*P<0.05, **P<0.01).

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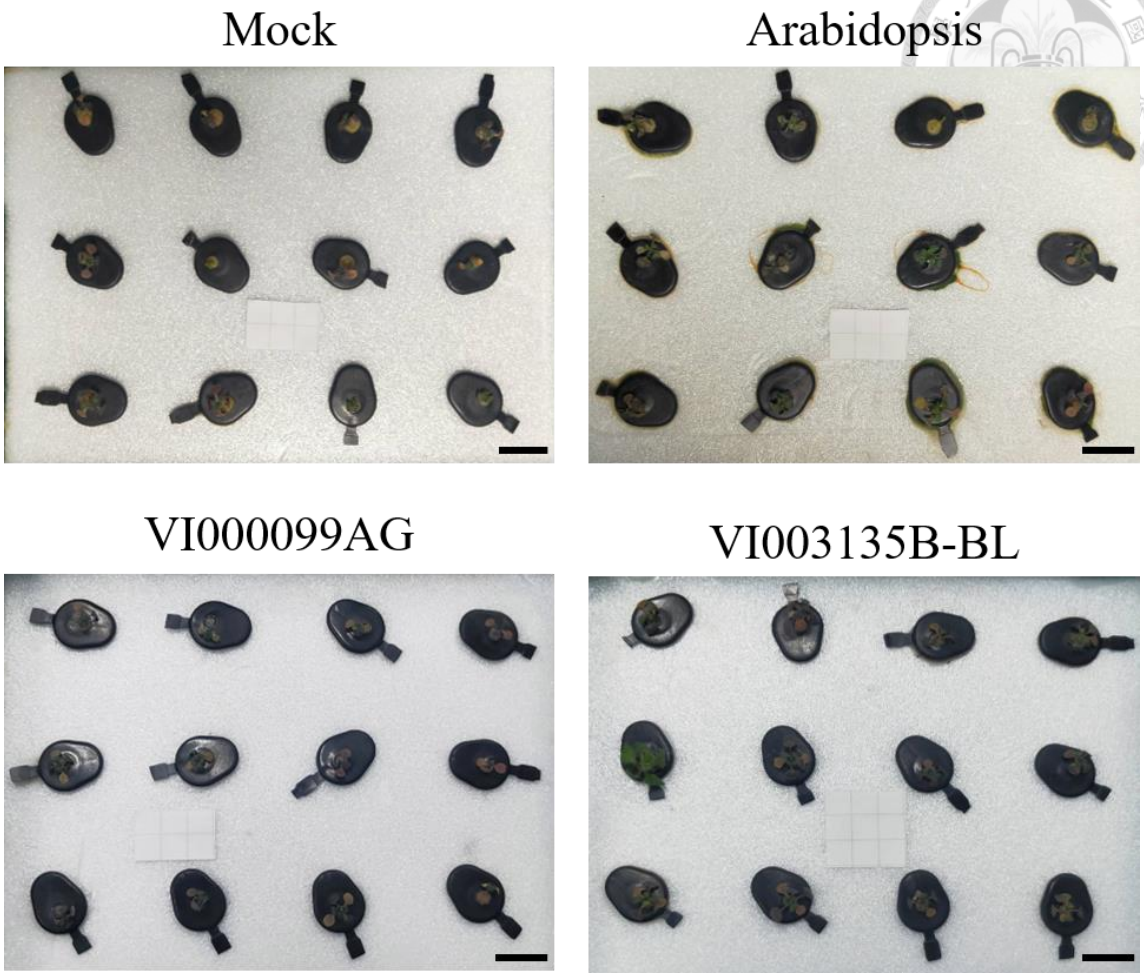

Figure 3.12 Hydroponic Arabidopsis subjected to 80% methanol-extracted mungbean metabolites

Analysis of rosette fresh weight in three-week-old wild-type hydroponic Arabidopsis plants subjected to metabolites from different mungbean cultivars (VI000099AG, VI003135B-BL) that were extracted with 80% methanol. The treatment of Arabidopsis extract was used as a positive control to show the inhibition activity in mungbean extract. Mock represented the negative control that the nutrient solution contains 1mL of 80% methanol. Scale bar, 1 cm.

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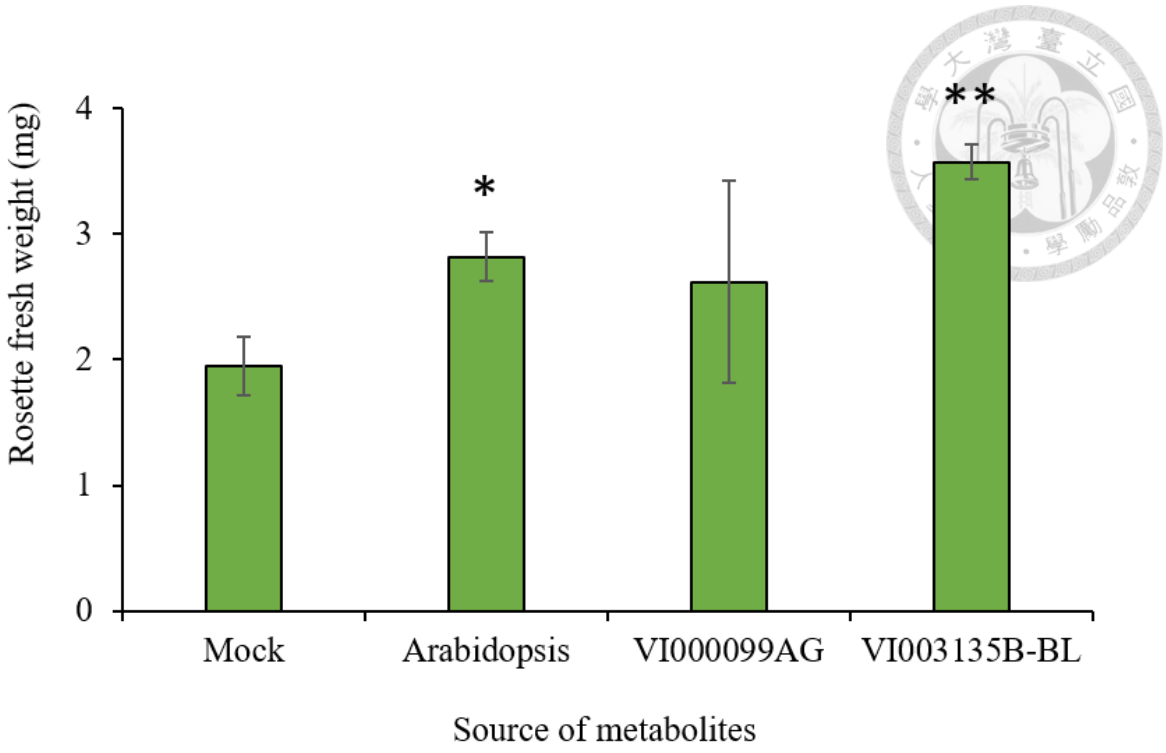

Figure 3.13 Hydroponic Arabidopsis rosette fresh weight subjected to 80% methanol-extracted mungbean metabolites

Analysis of rosette fresh weight in three-week-old wild-type hydroponic Arabidopsis plants subjected to metabolites from different mungbean cultivars (VI000099AG, VI003135B-BL) that were extracted with 80% methanol. The treatment of Arabidopsis extract was used as a positive control to show the inhibition activity in mungbean extract. However, the rosette fresh weight of all treatments was heavier than the mock. Three biological repeats were carried out. Each treatment has 11-12 plants. Mock represented the negative control that the nutrient solution contains 1mL of 80% methanol. Statistical significance has been estimated as compared with control using one-tailed Student's t-test. Asterisks represent a significant difference (*P<0.05, **P<0.01).

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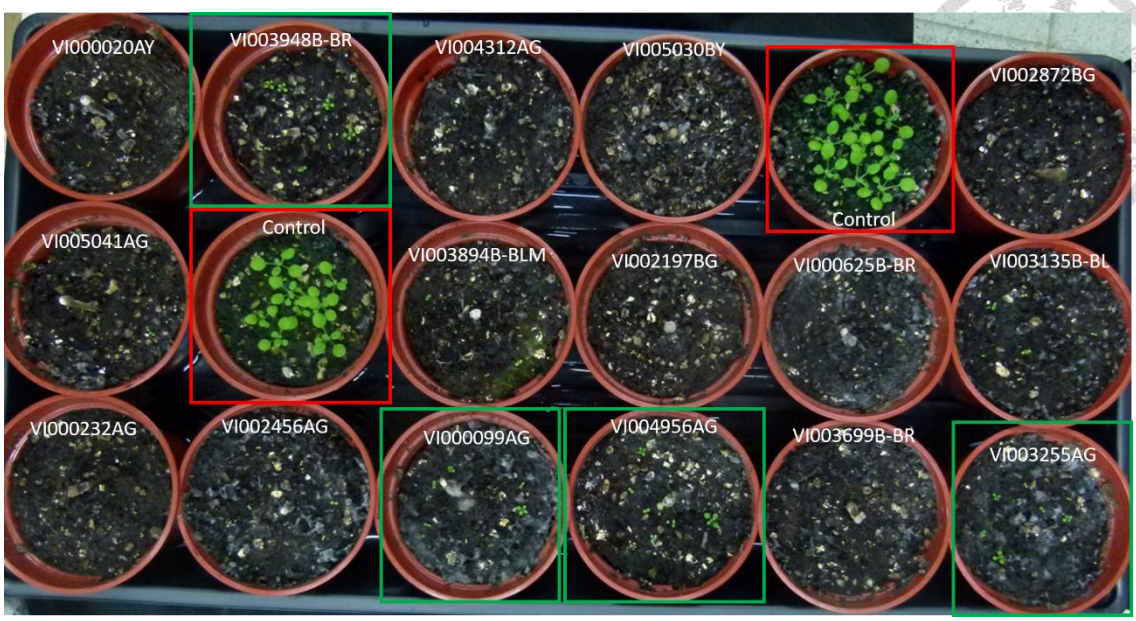

Figure 3.14 Comparison of inhibition effect on Arabidopsis growth between mungbean accessions

Arabidopsis growth on soil on which a mungbean accession grew before. Except for the control, the Arabidopsis plants were strongly inhibited. However, the extent of growth inhibition was slightly different among mungbean accessions. Control represents that the Arabidopsis plants grew on the soil never used to grow mungbean.

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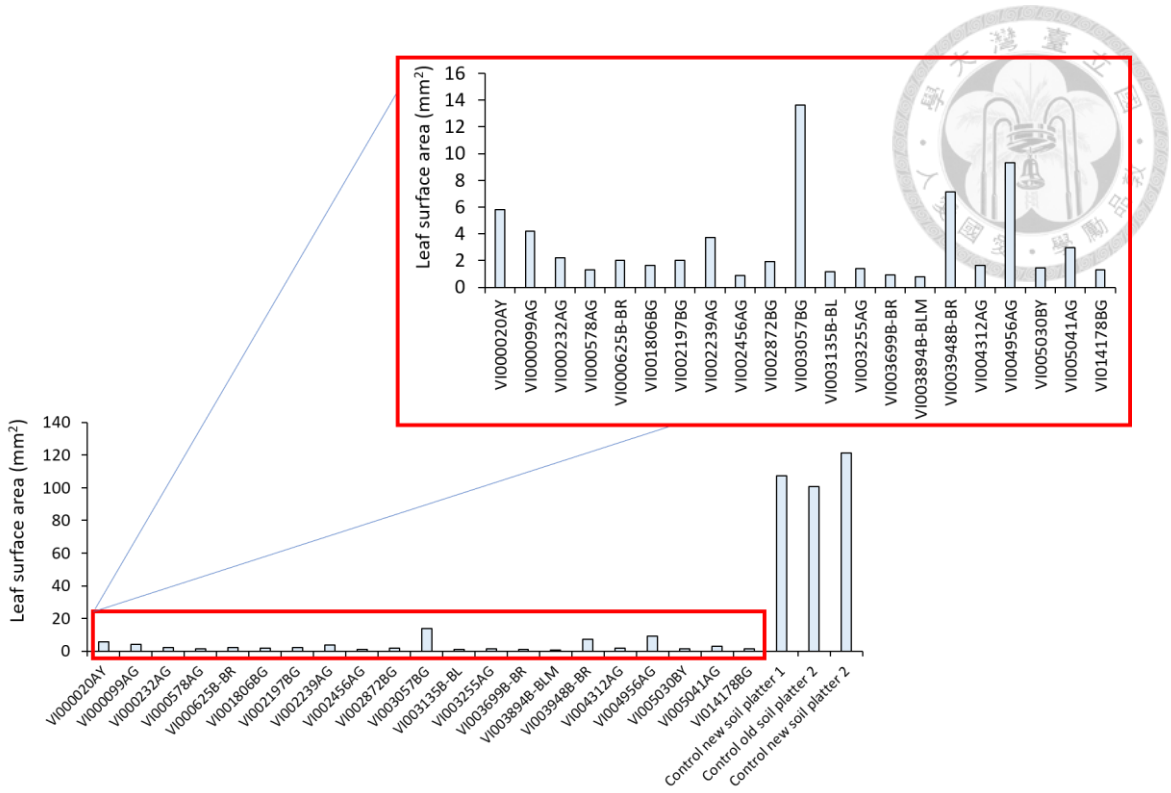

Figure 3.15 Measure of the leaf surface area of Arabidopsis plant grown in the pots after mungbean accessions

The leaf surface area of Arabidopsis plants under the inhibition effect from mungbean was much smaller than that of the control. Some variability of the leaf size was observed among mungbean accessions.

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4. Discussion

4.1 Feasibility of using L-DOPA for Fe biofortification is plausible

The application of natural products for biofortification instead of transgenic plants is a novel idea. In practice, it could be combined with other approaches, for example, conventional breeding. However, there are predictable obstacles that will be met in this project afterward. First, the effect of L-DOPA treatment would be influenced by the soil type. The reactions of adsorption, catalytic transformation, and biotransformation would lead to the disappearance of L-DOPA in soils. Volcanic ash, calcareous, and alluvial soils were compared on the ability to reduce the growth inhibition of L-DOPA in the previous study. The first order was calcareous soil owing to the highest pH value, volcanic ash soil came next because of its strong adsorption capacity to L-DOPA, and alluvial soil was the last (Furubayashi et al., 2005). Second, the practice of replacing exogenous purified L-DOPA with the root secretion of an L-DOPA-producing species intercropped with the target plant is economical but can lead to the problem that metabolites secreted by the intercropped plant are toxic to the target plant, as it is the case of mungbean in this study. Even L-DOPA itself is a potent allelochemical that suppresses the root growth of some species. Fortunately, our ultimate target of Fe biofortification, staple crops such as rice

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and wheat (*Triticum aestivum*) belonging to Poaceae, are less sensitive to L-DOPA (Nishihara et al., 2004). Although the aforementioned obstacles remain to be overcome, the first experiment already laid the groundwork for the project with a promising result that Arabidopsis seeds treated with L-DOPA contained more Fe. Moreover, in the study described in Zhan et al. (2016), the Cd-accumulating species *Sonchus asper* produced more biomass and accumulated more Cd in their leaves and stems when intercropped with fava bean, a L-DOPA-secreting species. The promising result was not linked to L-DOPA, but this is a likely explanation. This is an indication that metal uptake can be improved by allelochemicals secreted by intercropped plants.

4.2 The extraction method for analyzing mungbean on HPLC needs improvement

During the experiment in this study, two columns were blocked making the pressure rise consequently. Even little changes in system pressure can affect retention times and sensitivity for HPLC analysis, which causes the low concentration of L-DOPA in mungbeans to become undetected after the column blockage. To avoid blocking another column after the first one was broken, each sample was centrifuged and the supernatant was transferred to a new tube four times. The supernantant was then filtered through a

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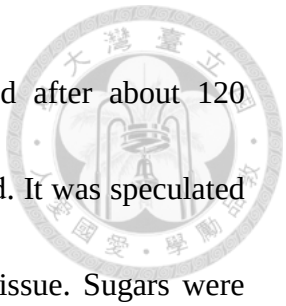

0.45 μm filter before injection. However, the problem reoccurred after about 120 injections of samples though the impurities were thoroughly removed. It was speculated that the high content of sugars in mungbean seeds may cause the issue. Sugars were extracted together with the L-DOPA and might precipitate in the column and clog it. The observation of precipitated substances that appeared in the samples when they were taken out of the refrigerator or left at room temperature for a week support this assumption. Mungbean seeds have a high content (50~60%) of carbohydrates with starch accounting for the largest proportion (Tang et al., 2014). To avoid damage from the mungbean seed matrix to columns, the preprocessing of samples before analysis must be optimized. Changing the extraction solvent should be a cost-effective way in contrast to removing the sugars from the extract solution. According to the reference (Vora et al., 2017), 50% methanol was demonstrated to produce good extraction efficiency. Additionally, given the low solubility of biomacromolecules in most organic solvents, the method may greatly reduce the interference from the matrix of mungbean with the optimized methanol proportion.

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4.3 Determining whether mungbean secrets L-DOPA into the soil

Although the mungbeans were found to contain L-DOPA in their seeds in the previous experiment (Fig. 3.5), it remains unknown if mungbean secretes this compound from the roots into the soil. Thus, the mungbean metabolites extracted from the potting soil growing mungbeans for 8 weeks will be analyzed by HPLC to find out the answer.

4.4 The allelopathic effects of mungbean in intercropping

Even though the effect of growth inhibition in Arabidopsis by mungbean is against the aim of the present study, it is a foreseeable outcome. Numerous crops, including mungbean, release allelochemicals that turn the soil toxic resulting in the reduction of subsequent crop yields. Indeed, allelopathy was estimated to contribute 10-25% to growth inhibition from continuous cropping of mungbean (Waller et al., 1994). Not only the root exudates but also the decomposed residues of mungbean are allelopathic. Allelochemicals from mungbean root and stem were identified by HPLC to contain compounds that have the peaks of the same retention time as saponin compounds, thioglycerol and aglycone (Lertmongkol et al., 2011). Nevertheless, different plants would have different

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sensitivities to the allelopathic effect of mungbean. When lettuce and *Echinochloa crus-galli* were grown in agar medium in Petri dishes with mungbean respectively, lettuce was inhibited in the germination and root length as the germination and the shoot growth were stimulated in the latter (Lertmongkol et al., 2011).

By far, the complex mechanisms of allelopathy among crops remain elusive. Factors of plant species, growth stages, and environments may all cause different allelopathic effects. In this study, it was shown that the ability of inhibiting Arabidopsis growth is variable among accessions. It is therefore possible to take advantage of the genetic resources available for mungbean to study the mechanisms of allelopathy in mungbean, and maybe even identify one or more allelopathic molecules.

Intercropping is a promising strategy to supply the L-DOPA to a target crop. However, the critical point is to select crops that do not negatively affect each other, which is usually determined by years of trial. *Sesbania cannabina* and sun hemp were analyzed in this study because they are commonly used as green manure in Taiwan and therefore have positive effects, rather than negative, on crop yield. Mungbean in practice is intercropped with the main crops such as maize, cotton, and rice (Bibi et al., 2020; Khan and Khaliq, 2004; Mandal et al., 1990). Based on this fact, mungbean remains a candidate for supplying L-DOPA to rice or maize, in order to increase their Fe content and as a consequence their nutritional value.

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5. Conclusion

Fe deficiency has gradually received more attention as an important health problem in recent years. With a better understanding of the transport and signal pathways of Fe in plants, scientists are devoted to the research of biofortification. The present study suggests that L-DOPA increase Fe content in Arabidopsis seeds while the yields were unchanged; 50 cultivars of mungbean were detected and confirmed to produce L-DOPA at the average concentration of 18.6 $\mu g.g^{-1}$, which is higher than the other tested legumes (adzuki bean, soybean, *Sesbania cannabina*, and sun hemp). However, Arabidopsis plants exhibited growth inhibition when co-cultivated with mungbeans. It is uncertain whether the several phytotoxic compounds found in the metabolites profile are associated with the allelopathic effects. For agricultural application purposes, more research is needed to assess whether L-DOPA has the same effect when applied in the field and whether mungbean can provide L-DOPA when intercropped with suitable target crops.

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Supplementary Tables

Supplementary table S1. Iron requirements of 97.5% of individuals in terms of absorbed iron[a], by age group and sex (World Health Organization, 1989)

Age/sex	in μg/kg/day	in mg/day[b]
4-12 months	120	0.96
13-24 months	56	0.61
2-5 years	44	0.70
6-11 years	40	1.17
12-16 years (girls)	40	2.02
12-16 years (boys)	34	1.82
Adult males	18	1.14
Pregnant women[c]		
Lactating women	24	1.31
Menstruating women	43	2.38
Post-menopausal women	18	0.96

[a] Absorbed iron is the fraction that passes from the gastrointestinal tract into the body for further use. [b] Calculated on the basis of median weight for age. [c] Requirements during pregnancy depend on the woman's iron status prior to pregnancy

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Supplementary table S2. List of 50 mungbean cultivars

Accessions number (Provenance of material)		
VI000020AY (Thailand)	VI001435AG (United States of America)	VI002647AG (Thailand)
VI000099AG (India)	VI001509AG (Pakistan)	VI002859BG (Iran)
VI000232AG (Iran)	VI001514AG (India)	VI002872BG (Iran)
VI000238AG (Afghanistan)	VI001728AG (India)	VI002934AG (India)
VI000542BY (India)	VI001806BG (Pakistan)	VI002986AG (India)
VI000578AG (India)	VI002176BG (India)	VI003057BG (India)
VI000625B-BR (India)	VI002197BG (Republic of Korea)	VI003135B-BL (India)
VI000938AG (India)	VI002239AG (Afghanistan)	VI003255AG (India)
VI001191BG (Philippines)	VI002456AG (Republic of Korea)	VI003337BG (India)

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VI003456AG	VI004184AG (Netherlands)	VI004973B-BLM (India)
VI003465BG (India)	VI004243B-BR (Turkey)	VI005022BG (India)
VI003480BG (India)	VI004244B-BR (India)	VI005030BY (Mexico)
VI003534BG (India)	VI004312AG (India)	VI005041AG
VI003699BG (India)	VI004432B-BR (Iran)	VI014178BG (Kenya)
VI003894B-BLM (India)	VI004666AG (Iran)	
VI003925B-BLM (India)	VI004853BG (India)	
VI003948BG (India)	VI004956AG (Pakistan)	
VI004069BG (India)	VI004965BG (Pakistan)	

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Supplementary table S3. The formula of ES media

Macronutrients	**Concentrations (mM)**
KH_2PO_4 (Merck, CAS-No: 7778-77-0)	2.5
KNO_3 (Merck, CAS-No: 7757-79-1)	5
$MgSO_4$ (Merck, CAS-No: 10034-99-8)	2
$Ca(NO_3)_2$ (Merck, CAS-No: 13477-34-4)	2
Micronutrients	**Concentrations (μM)**
H_3BO_3 (Merck, CAS-No: 10043-35-3)	70
$MnCl_3$ (Merck, CAS-No: 13446-34-9)	14
$CuSO_4$ (Merck, CAS-No: 7758-99-8)	0.5
$ZnSO_4$ (Merck, CAS-No: 7446-20-0)	1
Na_2MoO_4 (Sigma-Aldrich, CAS-No: 10102-40-6)	0.2
$CoCl_2$ (Sigma-Aldrich, CAS-No: 7791-13-1)	0.01
Fe-EDTA (made of $FeCl_3$ and EDTA) $FeCl_3$ (Merck, CAS-No: 10025-77-1) EDTA (Merck, CAS-No: 25102-12-9)	50

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Supplementary table S4. Settings of gradient and flow rate on HPLC

Time (min)	A (%)	B (%)	Flow (mL/min)
0	100	0	1.2
6	100	0	1.2
6.1	0	100	1.2
11	0	100	1.2
11.1	100	0	1.2
20	100	0	1.2

A: pH 2.0 H_2O adjusted with phosphoric acid

B: 70% methanol

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Supplementary table S5. Settings of gradient and flow rate on LC-MS

Time (min)	A (%)	B (%)	Flow (μL/min)
0	0	100	350
2	0	100	350
7.7	30	70	350
9.5	60	40	350
10.25	70	30	350
12.75	0	100	350
16.75	0	100	350

A: 10 mM ammonium formate in H_2O + 0.1% formic acid

B: 10 mM ammonium formate in 95% ACN/5% H_2O + 0.1% formic acid

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Supplementary table S6. Settings on mass spectrometry

Instrument:	BrukertimsTOFPro®	
Source:	ESI positive and negative	
Capillary	4500V (+) 4200 (-)	
Nebulizer	2.5 bar	
Dry Gas	10 L/min	
Dry Temp	250°C	
MS settings:	3D-Metabolomics (DDA MS/MS)	4D-Metabolomics (tims-PASEF MS/MS)
Mass Range:	20–1300m/z	20–1300m/z
Ion Mobility Range	-	0.45–1.45 V*s/cm2
Ramp time	-	100ms
MS/MS settings:		
Total Cycle Time	0.5 sec	0.5 sec
Number of MS/MS ramps	-	60 PASEF scan
Collision Energy	20 eV; 50 eV	20 eV; 50 eV
Intensity Threshold	350	100
Active Exclusion	3	On

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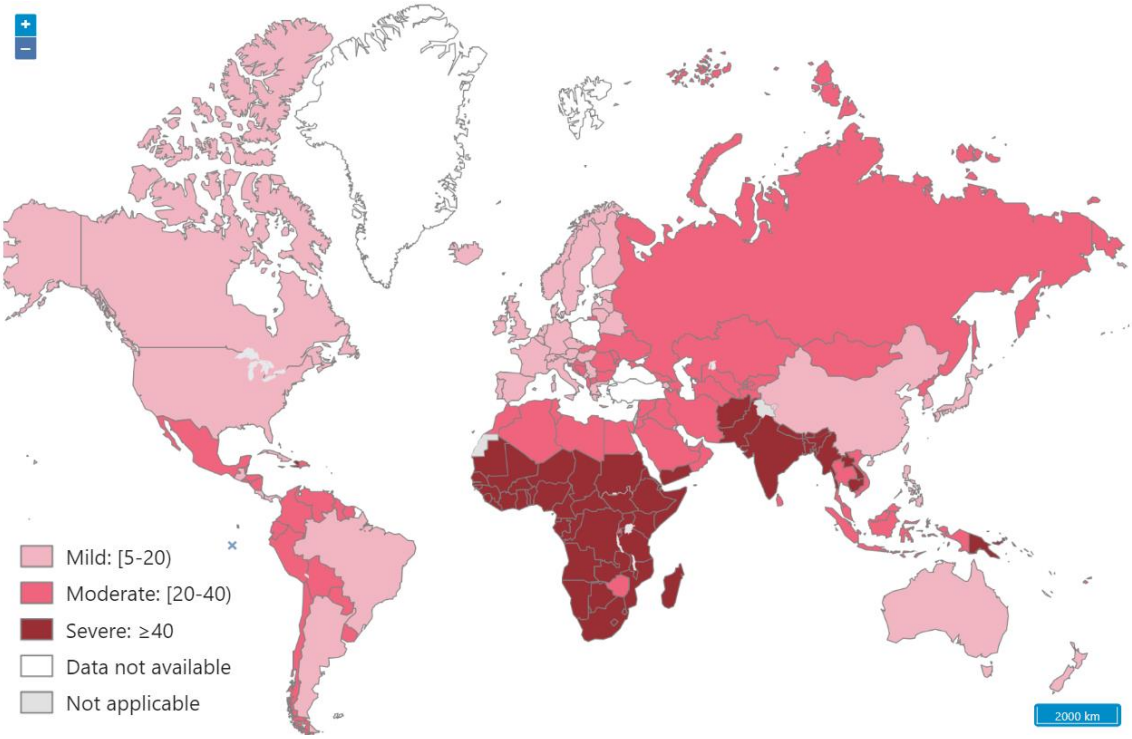
Supplementary Figures



Supplementary Figure S1. Prevalence of anemia in children aged 6 months to 5 years in the world

Source: WHO database on anemia

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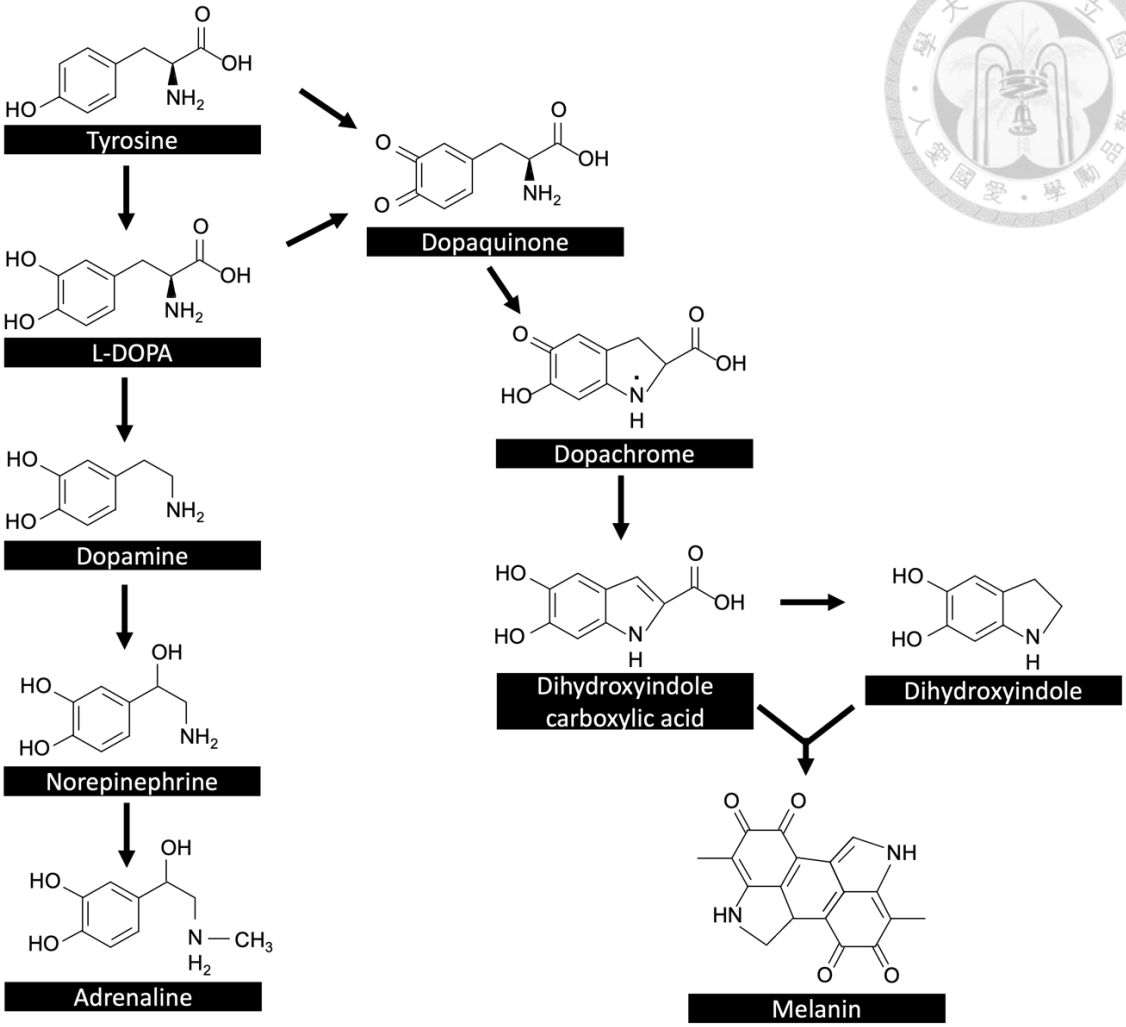

Supplementary Figure S2. L-DOPA metabolic pathway

L-DOPA is the precursor of the melanin pigments and the catecholamine neurotransmitters, dopamine and adrenaline, in humans.

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Supplementary Figure S3. Comparison of growth inhibition in non-treated and L-DOPA-treated soybean seedlings (Soares et al., 2011)

Inhibitory effect of L-DOPA on the growth of mungbean at germination, and formation of black compounds in the roots of treated plants.

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