INVESTIGATON OF FACTORS INFLUENCING GROWTH AND OXYPINNATANINE ACCUMULATION AND SECONDARY METABOLITES IN DAYLILY (*Hemerocallis* spp.)

Daylily（*Hemerocallis* spp.）の生育およびオキシピナタニン含量に影響を与える要因の分析および同属植物における二次代謝物のスクリーニングおよび抗酸化能測定

NGUYEN THI HONG NHUNG

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Abstract

Daylily (*Hemerocallis* spp.) is a perennial medicinal plant, having been utilized for thousands year in Asia to treat some diseases. The factors influencing growth, yield and oxypinnatanine (OPT) accumulation, the phytochemical components, and DPPH radical scavenging ability of daylily were investigated in this research.

The evaluation of growth, yield and OPT accumulation of *H. fulva* var. *sempervirens* (Synonym: *H. sempervirens)* cultivated in dark-red, gray, and red soils of Okinawa, Japan and in dark red soil with eight of N, P, K amount combinations (0.0 – 0.6 g/pot, size 1/5000a) revealed that dark-red soil was better for plants to grow, yield, and accumulate OPT than the other soils. In dark red soil, growth and leaf yield of *H. sempervirens* were proportional to supplied N,P,K amount, but flower yield and OPT content were not. The N,P,K combinations at 0.4 – 0.6 g/pot promoted the leaf yield; at 0.4 g/pot was the best for production of flower; and at 0.3 – 0.4 g/ pot were ideal for the OPT accumulation.

Besides, this research also pointed out that environmental factors such as relative light intensity (RLI) and temperature conditions influenced markedly OPT accumulation of *H. sempervirens*, but waterlogged soil did not. The plants grown under condition of 100% and 40% RLI (non-shade and severe shade, respectively) produced significantly more OPT than those of 75% and 60% RLI. Plants exposed to 40°C for 10 days before harvesting accumulated the most OPT, followed by to 15°C in comparison with to range of 20 – 35°C. The lowest OPT amount was observed in plants treated at 10°C. In Okinawa, *H. sempervirens* accumulated the highest OPT concentration in December and January in comparison with the rest months in a year, next

by in August and September. After harvested, *H. sempervirens* leaves could be stored for 2 days at room temperature, 10 days at 0 – 4ºC, and 150 day at –20ºC to preserve OPT content. Store method of drying at 50ºC made the decrease about 75% of OPT in leaves.

The analysis of relationship between some internal factors and OPT accumulation of 24 daylily strains revealed that ploidy level (2x and 3x) did not influence OPT synthesis. However, it was observed that of 16 strains of the same species *H. fulva*, 14 triploid strains showed higher OPT content than 2 diploid strains. Genetic diversity and growth stage affected significantly on OPT accumulation of daylily: the different strains in the same species, the different species, and the different genetic similarity groups showed the different OPT concentration; daylily plants during the vegetative and flowering periods produced more OPT than during the post-flowering period.

The qualitative analysis of 13 main phytochemical compounds in methanol daylily extracts found the presence of 12 compounds. The high content of total phenolic (77.80 – 317.13 mg GAE/g extract) and flavonoid (15.96 – 33.00 mg CE/g extract) had a positive correlation to antioxidant capacity of daylily extracts. These results may provide an explanation for many medicinal benefits of daylily and indicate a value source of bioactive compound for pharmacological industry.

Abstract in Japanese

ヘメロカリス属（デイリリー）はいずれも多年生の薬用植物であり、アジアでは以前から様々な病気の治療に用いられてきた。本研究では *Hemerocallis sempervirens* の生育、およびオキシピナタニン含量について様々な条件下での差異を調査するとともに *Hemerocallis* 属植物の二次代謝物、抗酸化能などについて調査を行った。

沖縄県の三つの代表的な土壌種、島尻マージ, 国頭マージおよびジャーガルにおける *H.sempervirens* のポット栽培での生育は島尻マージ、ジャーガル、国頭マージの順に高い値を示した。N, P および K 肥料をそれぞれ 0、0.1、0.2、0.3、0.4、0.5、0.6 g/pot（1/5000a）で等量ずつ施用し、葉重、花重およびオキシピナタニン含量を測定した結果、葉重は 0.4、0.5、0.6 g、花重は 0.4 g、オキシピナタニン含量は 0.3　g および 0.4 g の施用で高い値を示した。*H.sempervire* を冠水土壌条件および非冠水の土壌条件で栽培し、オキシピナタニン含量を測定したところ、両者間には差が見られなかった。一方、日照量が 100%、75%、60%および 40%条件の遮光効果実験においては、遮光を施さない 100%日照量および強い遮光の 40%日照量でオキシピナタニン量が増加した。10℃、15℃、20℃、25℃、30℃、35℃および 40℃に 10 日間置いた後、オキシピナタニン量を測定した結果、40℃で最も高く、続いて 15℃で高かった。10℃では最も低い値を示し、25℃から 35℃では中間的な値を示した。収穫葉を室温で保存し、その後オキシピナタニン量を一日単位で測定した結果、2 日目までは収穫時との間で差がなかったが、3 日目以降は徐々に低下した。冷蔵保存（0 － 4℃）では１０日までは収穫時との

差はなかったが、その後は低下していった。－20℃の保存では 150 日頃まではオキシピナタニン 量の低下は見られなかったが、その後は低下が見られた。ただし、低下の程度はそれほど大きくなく、最後に測定した 240 日でもある程度のオキシピナタニン量を保持していた。収穫葉を 50℃で乾燥させた場合、オキシピナタニンは大部分が消失していた。

世界各地から収集した 4 種 24 系統はいずれもオキシピナタニンの存在が認められた。ただし種および系統によって含有量は異なっていた。調査材料では 2 倍性と 3 倍性の間に差は認められなかったが、*H.fulva* の 2 倍性系統と 3 倍性系統で比較したところ、3 倍性で有意に高いかまたは高い傾向にあった。

Hemerocallis 属の 3 種を用いてフェノール類、テルペン類および含窒素化合物の二次代謝物質をスクリーニングしたところ、いずれの種でも調査した 13 種のうち 12 種の存在が確認できた。総フェノール含量 77.80 － 317.13 mg、総フラボノイド含量 15.96 － 33.00 mg であり、種間差はあったもののいずれも高い値を示した。総フェノール含量および総フラボノイド含量と DPPH に対する抗酸化力の間には正の相関が見られた。

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Contents

CHAPTER 1

General Introduction

Plants used in traditional medicine have played an important role in treating diseases and improving health throughout human history. The rich and diverse source of bioactive secondary metabolites from these plants has been value resources for discovering and developing modern drugs by the pharmaceutical industry.

Daylily (*Hemerocallis* spp.*, Hemerocallidaceae*) is a herbaceous perennial plant. It is native to Asia throughout China, northern India, Japan and Korea. But nowadays geographical distribution of daylily has been extended much, for examples, it is presence in North America (Ehrenfeld, 2008), European (Webb, 1980, Pyek, 2003). In China, Korea, and Japan, daylily is used both as medicine for treatments of depression, inflammation, jaundice, and liver disorders and as food items (Shanghai Scientific Technological Publishers and Shogakukan, 1985, Jiangxi Medical College, 1986, Tobinaga, 1999, Jennifer, 2011). Daylily is also used as a pain reliever, a diuretic, an antidote to arsenic poisoning and an anticancer agent (American Hemerocallis Society, 2007). Besides, daylily has been reported to possess antioxidant activities, to be active against the human pathogenic trematode *Schistosoma mansoni* (Cichewicz and Nair, 2002, Cichewicz *et al.*, 2002, Zhang *et al.*, 2004), and to inhibit some lines of human tumor cell (Cichewicz *et al.*, 2004).

Hemerocallis fulva var. *sempervirens, (*synonym: *H. sempervirens)* a member of genus *Hemerocallis* has been used as a traditional therapy to cure insomnia in Okinawa, Japan since

long time ago. The recent reports have showed that chemical constituent possessing the sedative and sleep-improving effects of this species is oxypinnatanine (OPT), an N-glycoside structure amino acid amide that is unique derived of glutamic acid with a furfuryl group. (Uezu, 1998, Uchiyama *et al.*, 2007, Ogawa and Konishi, 2009, Urade *et al.*, 2010, Ogawa *et al.*, 2013).

Recently, in Okinawa, area of commercial *H. sempervirens* cultivation has been expanded in order to provide materials for the production of OPT-containing sleep aid products such as tea and capsules (Yoshihara *et al.*, 2011). However, until now, there are no good cultivation methods for this plant. Also, there is no information regarding the effects of internal and external factors on *H. sempervirens* and its OPT accumulation, although there are many reports on sedative and sleep-improving effects of crude extract as well as of OPT isolated from *H. sempervirens*. Besides, studies on the presence of OPT and sleep-improving effects of daylily have been only conducted with some members of species *H. fulva,* such as *H. sempervirens, H. fulva* var. *fulva, H. fulva* var. *longituba, and H. fulva* var. kwanso (Uezu, 1998, Konishi *et al.*, 2001, Ogawa and Konishi, 2009, Yoshihara *et al.*, 2011). Consequently, in this thesis, influences of factors on growth, yield and OPT accumulation of *H. sempervirens* as well as the presence and concentration of OPT in some daylily species and cultivars were investigated. Based on the obtained results, the efficient cultivation procedures for daylily, especially *H. sempervirens* and the methods for improvement of the OPT quantity in daylily could be established.

As mentioned, daylily has been used as traditional medicine therapeutics not only to cure imsomnia but also to treat inflammation, depression, and jaundice etc. In addition, recently pharmacological studies has demonstrated pharmaceutical activities of dayily that may be applied to treat many chronic diseases such as cancer, cardiovascular diseases, arteriosclerosis

and aging related disorders (Cichewicz and Nair, 2002, Cichewicz *et al.*, 2004, Zhang *et al.*, 2004). These benefits indicate the large pharmacological potential of daylily. The medicinal properties of plants are based on the presence of secondary metabolite constituents. Thus, the knowledge of the secondary metabolite profile of daylily would further be valuable in discovering the actual medicine values of this plant. In this study, the medicinal potential was preliminarily investigated through screening phytochemical constituents and antioxidant capacity of some daylily strains. The followings are main issues investigated in this dissertation:

1. Influences of soil types in Okinawa Japan and N, P, K fertilizations on growth, yield, and OPT concentration in *H. fulva* var. *sempervirens.*
2. Effects of some environmental factors such as waterlogged soil, relative light intensity, temperature, harvesting time, and storage methods for post-harvested leaves on OPT concentration in *H. fulva* var. *sempervirens* in Okinawa.
3. Relationship between ploidy level, genetic diversity, growth stages and OPT accumulation in daylily.
4. Secondary metabolite constituents and antioxidant capacity of some daylily strains.

CHAPTER 2

Influences of Three Soil Types in Okinawa, Japan and N, P, K Fertilizations on Growth, Yield and Oxypinnatanine Accumulation of *Hemerocallis fulva* var. *sempervirens*

2.1. Introduction

Hemerocallis fulva L. var. *sempervirens* (synonym: *H. sempervirens)*, a member of genus *Hemerocallis*, distributes in Kyushu and Okinawa islands of Japan. This is a perennially medicinal plant, having flowering season within August to November (http://flowers3.la.coocan.jp/Hemerocallidaceae/Hemerocallis%20fulva%20sempervirens.htm). *H. sempervirens* has been used as a folk remedy to cure insomnia in Okinawa since ancient time (Uezu, 1998). Yoshihara *et al.* (2011) reported that 64% people taking part his experiment said that *H. sempervirens* improved their sleep time and physical conditions in next day based on the visual analog scale; 60% people felt better in vitality in next day; average sleep time of people taking *H. sempervirens* was 0.7 h more than their sleep time in one month before, and 1.1 h more than that of people taking placebo. Uchiyama *et al.* (2007) demonstrated that after oral administration at the dose of 300 mg of *H. sempervirens* extract per kg of mice body weight, *H. sempervirens* extract reduced the locomotor activity of mice for 5 hours. Some pharmacological studies have demonstrated that oxypinnatanine (OPT) (Fig. 2.1), a unique derivative of glutamic acid or glutamine with a furfuryl group, is the major active component of this plant for sedative

and sleep-improving effects. Urade *et al.* (2010) showed that at a dose of 100 mg/kg, OPT significantly increased Non-Rapid Eye Movement (NREM) sleep time in mice in the 6 hours after the administration compared to the control, on the other hand OPT decreased maintenance of wakefulness, sped up the transition from wakefulness to NREM sleep and increased NREM sleep time. Also, Ogawa *et al.* (2013) indicated that at an oral dose of 30 mg/kg, OPT increased the total time of NREM sleep by 84% in mice in the 3 hours after administration.

Recently, the commercial cultivation of *H. sempervirens* has increased in Okinawa, for the production of OPT-containing sleep aiding products such as tea and capsules (Yoshihara *et al.*, 2011). However, the cultivation method for this plant has not established yet. Almost no information regarding the effects of cultivation factors and effective cultivation technologies for this plant and its OPT has been reported. As other plants, the growth, yield and quality of *H. sempervirens* are affected by a host of external factors such as type and composition of soil, kinds and balance of nutrients, light intensity, temperature, water availability, and others. Soil and nutrients are certainly among the most important factors for all plants. Different plants grow well and produce high yields and quality in different soil types and nutrient levels. A study on the effects of these factors on plant cultivation under local climatic and edaphic conditions is necessary to gain a high yield with a high quality. This study was designed to evaluate the influence of three main soil types, dark-red soil, gray soil and red soil, in Okinawa, Japan and quantitative combinations of N, P, K fertilizers on growth, yield and OPT accumulation of *H. sempervirens*.

2.2. Materials and Methods

2.2.1. Plant materials

H. sempervirens plants preserved in the experimental fields of the Faculty of Agriculture, University of the Ryukyus, were used as the plant materials in this study. The plants were pruned and cultivated in experiments of soil types and N, P, K fertilizations. Pruned plants had similar weight of 200 g, 6 to 7 cm in height, with 4 to 5 roots. Each root was about 5 cm in length.

2.2.2. Soil types

Dark-red and gray soils were collected from the fields of the Subtropical Field Science Center, University of the Ryukyus, Okinawa, Japan at a depth of 50 cm. Red soil was collected from the hilly region in Ishikawa, Okinawa, Japan at the same depth. Nitrate nitrogen (NO_3-N) and ammonium nitrogen (NH_4-N) of soils were extracted by 2M KCl solution as descriptions of Maynard *et al.* (2006). Then NO_3-N and NH_4-N content were determined by AACS III analyzer (Bran + Luebbe, A United Dominicom Co. Germany and Japan). The other nutrient elements contained in the soils were extracted by Mehlich-3 solution as descriptions of Ziadi and Tran (2006). Concentrations of these elements in extracts were determined using an Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES, ICPE-9000, Shimadzu Co. Ltd.). Total carbon was measured by using NC analyzer (Sumigraph NC-220 F, SCAS Co. Ltd.). The physical properties of soils including content of sand, silt, and clay, and bulk density were determined using methods described by Arnold (1986). The soil pH was measured from the soil water suspension at 1:2.5 ratio of soil to deionized water. Results of the determination of physical and chemical properties of dark-red, gray and red soils are presented in Table 2.1.

2.2.3. N, P, K fertilizers

Applied fertilizer sources of N, P, K elements were ammonium sulfate (21% N), granular super phosphate (17.5% P_2O_5), and potassium chloride (60% K_2O).

2.2.4. Plant cultivation for experiments

Experiment of effects of soil types on H. sempervirens

The experiment was conducted between June 2011 and December 2012. *H. sempervirens* was cultivated in Wagner pot (size 1/5000a), filled with about 3 kg of air-dried corresponding soils. Each pruned plant took a pot. Thirty pots were used for each soil, and they were placed complete randomly in a net house. Plants were supplied about 0.4, 0.4, 0.4 g/pot of N, P_2O_5, K_2O, respectively. Water was applied adequately for plants to grow and multiply.

Experiment of effects of N, P, K fertilizations on H. sempervirens

This experiment was also conducted between June 2011 and December 2012 in Wagner pots (size 1/5000a) with eight different combinations of N, P, K amounts. They were F1: 0.6, 0.6, 0.6; F2: 0.6, 0.5, 0.5; F3: 0.6, 0.4, 0.4; F4: 0.4, 0.4, 0.4; F5: 0.4, 0.3, 0.3; F6: 0.3, 0.3, 0.3; F7: 0.3, 0.2, 0.2; and F8: 0, 0, 0 (g/pot N, P_2O_5, K_2O, respectively. The amounts of fertilizer by 0.2, 0.3, 0.4, 0.5, 0.6 g/pot were the equivalent of 100, 150, 200, 250, 300 kg/ha, respectively). Each treatment consisted of ten pots. The whole dose of P and K and half the amount of N were applied at the time of planting. The remaining quantity of N was applied when plants finished dormancy period and rose 2-3 new leaves (about late January). Dark-red soil was used for this test because this soil was loose, and did not become waterlogged when supplied with water. Plants were watered adequately throughout the experimental period.

2.2.5. Investigation of OPT concentration in leaves and flowers of *H. sempervirens*

All of root, leaves, and flowers of *Hemerocallis* spp. have been used in treatment of some human diseases. But its roots showed more toxicity. Thus, mainly harvested and used parts are

flowers and leaves (Jiangxi Medical College, 1986). This is the reason why this study only focused on investigation of OPT compound in leaves and flowers of *H. sempervirens*.

The flowering season of *H. sempervirens* in Okinawa, Japan often lasts from about late August to November. And this experiment was carried out within September to December 2013. From the *H. sempervirens* fields of the Faculty of Agriculture, University of the Ryukyus, the 20 blooming plants were selected randomly for investigation of OPT concentration in leaves and flowers. From 10 of 20 selected plants, the flowers and leaves of each plant were gathered simultaneously to analysis OPT amount. The sampling time was throughout flowering season, namely 3, 4, 3 plants were sampled in September, October and November 2013, respectively. The flowers of the 10 remaining plants were harvested in period of concentrated blooming (early October 2013), and the leaves of these plants were sampled at the time of finishing flowering season (early December 2013) to determine OPT concentration.

2.2.6. Collection of samples to determine OPT concentration

For the investigation of the OPT concentration in flowers and leaves of *H. sempervirens*: From each plant of the 20 selected plants, whole fresh leaves and 2–3 fully opened flowers were sampled to analysis the OPT amount. The sampling time was during flowering stage and at the time of finishing blooming season as above descriptions.

For determination of OPT concentration in leaves of *H. sempervirens* in experiments of soil types and N, P, K fertilizations: From each treatment, three samples including whole the fresh leaves of three pots collected randomly were harvested to determine OPT concentration. The sampling was conducted at the same time of collecting the data of growth and leaf yield parameters (December 2012).

2.2.7. Determination of OPT concentration

Extraction of OPT from leaves and flowers of H. sempervirens

Extraction of OPT from *H. sempervirens* was carried out as descriptions of Ogawa and Konishi (2009) with minor modifications. Briefly, samples of *H. sempervirens* fresh leaves (FL) and flowers were cut into small pieces (approx. 2 mm × 5 mm) and extracted by 50% aqueous methanol at room temperature with shaking for 72 hours. Then they were filtered through No.6 Whatman filter paper. The extracts were concentrated using a vacuum rotary evaporator system, equipped with a rotavapor R-210, a vacuum controller V-850, a vacuum pump V-700, a heating bath B-491 (all from Büchi Labortechnik, Flawil, Switzerland) and a cool man PAL C-330 (from Sibata Co., Saitama, Japan). Then the extract residues were re-dissolved by a solvent containing 85% acetonitrile and 0.1% formic acid and filtered through a membrane filter (13 mm × 0.45 μm) into vials for analyzing.

Determination of OPT concentration

The OPT concentrations in sample extract solutions were determined by the Ultra Fast Liquid Chromatography (UFLC) system of Shimadzu Corporation, Japan. This system is equipped with an SPD M-20A prominence diode-array detector, two pumps of LC-20AD prominence liquid chromatography, a CTO-20AC prominence column oven and LCsolution software, also from Shimadzu. A Luna ®HILIC column (5 μm particle size, 150 mm length × 4.60 mm internal diameter) was utilized for the separation of OPT. The mobile phase consisted of Milli-Q water containing 0.1% (v/v) formic acid pH 3.5 for pump A, and acetonitrile with 0.1% (v/v) formic acid for pump B. The HPLC analysis was run with conditions of 85% B concentration, 1.0 mL/min flow rate, 10 µL injection volume and a column oven temperature of

40 – 45°C. The peak area of OPT was recorded at 200 nm UV. The different concentrations of OPT standard solution (100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 µg/mL) were analyzed to make a standard calibration expressing the correlation between peak area (y) and OPT concentration (x) in the corresponding analyzed solution. The concentrations of OPT in sample extracts were determined based on this curve. And the OPT concentrations in leaves and flowers were calculated by following formula:

$$C = \frac{\mathrm{Co} \times \mathrm{V} \times \mathrm{D}}{1000 \times \mathrm{W}}$$

where C is the OPT concentration in the leaves and flowers (mg/g), Co is the OPT concentration in the sample extract (µg/mL), V is the volume of the sample extract (mL), D is the dilution coefficient (if yes) and W is the weight of the sample (g).

2.2.8. Collection of growth and yield data

The parameters of growth and leaf yield of *H. sempervirens* in this study were recorded as soon as finishing of the flowering stage in 2012, (early December 2012).

Growth parameters consisted of (1) Plant height, measured from the soil surface to the arch of the uppermost leaf being at least 50% emerged from the top of plant; (2) Number of tillers per pot, referred to all plants emerged from original planted one in a pot; (3) Number of leaves per plant (dead leaves were not counted); and (4) Leaf biomass, the average weight of fresh leaves per plant, it was determined by ratio of leaf yield and number of plants per pot.

The yield consisted of (1) Leaf yield, expressed by the total fresh leaf biomass per pot and (2) Flower yield, referred to parameters of number of flowers per pot, recorded throughout

flowering season (late August to late November 2012), and flower weight, calculated by the average weight of 10 randomly collected flowers in each treatment.

2.2.9. Statistics analysis

The experiments were completely randomized designs. Results are expressed as means ± standard errors (SE) of parallel measurements. The SE and R values were determined by normal excel program. The means and differences between the treatments were determined by balanced analysis of variance (ANOVA) method of IRRISTAT program version 5.0. The values of least significant difference (LSD) was obtained at $P<0.05$.

2.3. Results and Discussion

2.3.1. OPT concentration in leaves and flowers of *H. sempervirens*

The OPT compound was accumulated in both leaves and flowers of *H. sempervirens*. The concentration of OPT in flowers (3.20 mg/g fresh flower) was significantly higher than that in leaves. The leaves at the time of finishing flowering stage accumulated more OPT (2.06 mg/g) than those during period of flowering (1.60 mg/g) (Fig. 2.2A). The OPT concentration in flowers and in leaves showed the strong positive relationship by the high values of linear correlation coefficient ($R=0.908$ and $R=0.899$, $P<0.05$) (Fig. 2.2B and 2.2C). Also, the regression equations showed the high coefficient of determination ($R^2= 0.8241$ and $R^2= 0.809$), that means it was possible to evaluate the OPT concentration in flowers from the OPT concentration in leaves, and vice versa.

There are many secondary metabolites which have been found in plants. They are thought to play a basic role in adaption to the environment and overcoming stress conditions of plants (Kliebenstein, 2004, Massad *et al.*, 2011). Content of each compound varies with different parts

of particular plant species, growth stage of plant, as well as specific environmental conditions. Ksouri *et al.* (2008) showed that in the species *Tamarix gallicai* content of phenolic compounds in leaves was three times lower than that in flowers, but in *Salsola kali* plants, content of this secondary metabolite in leaves was about 6-folds higher than that in flowers. Janska *et al*. (2010) indicated that all both high and low temperature levels resulted in increase in concentration of secondary metabolite to maximize tolerance of plants.

OPT is a N- based secondary metabolites, isolated from a few plants, such as *Euscaphis japonica* (Grove *et al*., 1973), *Honkenya peploides* (Cerantola *et al*., 2005), and *Hemerocallis* species (Ogawa *et al*., 2013). And *H. sempervirens* is a semi-dormancy plant. Like the other daylily strains, dormancy stage of *H. sempervirens* in Okinawa, Japan are about from middle December to early January. But different from some other strains, that take flowering stage within about April to August, *H. sempervirens* takes blooming season from about late August to late November. Thus it is assumed that the getting overwinter period as soon as finishing the flowering stage of this plant resulted in the higher OPT concentration in leaves at this time in comparison with that in leaves in the blooming season (Fig. 2.2A).

2.3.2. Effects of soil types on growth, yield, and OPT concentration

The measurements of growth, yield parameters, and OPT concentration in leaves of *H. sempervirens* cultivated in dark-red soil, gray soil, and red soil are shown in Table 2.2. Differences were observed for all measured parameters. The experimental results indicated that dark-red soil was the better choice for cultivation of this plant in comparison with the two other soil types. Almost all parameters of growth, such as plant height, number of leaves, and especially number of tillers of plants growing in dark-red soil were significantly higher than

those in gray and red soils. Thus, although the parameter of leaf biomass in dark-red soil (6.9 g/plant) was not statistically different from that in gray soil (6.2 g/plant), the leaf yield in dark-red soil (25.7 g/pot), was markedly higher than that in gray soil (18.5 g/pot), and in red soil (17.2 g/pot) (Table 2.2). The flower yield of *H. sempervirens* in this study was referred to the parameters of number of flowers and flower weight. The recorded data showed that number of flowers and flower weight produced by dark-red soil (8.7 flowers/pot and 3.1 g/flower) were clearly higher than those created by red soil (4.1 flowers/pot and 2.9 g/flower), but not clearly higher than those in gray soil (5.7 flowers/pot and 3.0 g/flower) (Table 2.2). Between gray soil and red soil, all parameters of growth and yield, except the parameter of plant height of plants in gray were not significantly different from those in red soil. The concentration of OPT in leaves was not proportional to the status of growth of the plant, for examples, although growth and yield of plants in red soil were the lowest, and those in dark-red soil were the highest, OPT concentration in plants growing in two these soils was not different. Both they reached 2.2 mg/g, significantly higher than that in gray soil (1.9 mg/g) (Table 2.2).

The soil affects complicatedly on plants through its physical, chemical and biological properties. It is not easy to interpret the influences of separate factors (Hornok, 1992). Each crop species grows well in a particular soil type with its kind and balance of nutrients. Of the three soils used in this study, the dark red soil had a neutral pH level of 6.4, whereas the gray and red soils were alkaline with pH at 7.3 (Table 2.1), respectively. Along with a neutral pH level, the loose structure (the lowest bulk density) of dark-red soil, and its soil texture, consisting of high clay (59.3 8%) and low sand (12.4%) contents, which are good for maintaining moisture and retaining nutrients, likely promoted the better growth and higher yield of *H. sempervirens* as

compared to the other soils. The red soil had not only the highest bulk density (0.92 g/cm^3), but also a particle size distribution of the highest sand content (44.5%) and the lowest clay content (28.7%). These physical properties made red soil become waterlogged for some time after watering and so compacted when dried, resulting in poor aeration and microbial activities, which probably restricted the growth and production of plants. About gray soil, except pH was the same as that of red soil, the other determined physical characteristics were just like intermediate of dark-red soil and red soil. It is assumed that these properties resulted in that growth and yield of plants in gray soil were better than that in red soil and lower than that in dark-red soil. These results are in agreement with the reports of Pennisi (2004) that daylilies could grow in a variety of soils, ranging from light soils to heavy clays, but that poorly drained soils are bad for daylily cultivation, and a mildly acidic soil, with a pH range of 6.0 – 6.5, is ideal for this plant. Hossain and Ishimine (2005) showed that the lowest apparent density and optimum moisture of dark-red soil resulted in greater growth of turmeric as compared to gray and red soil. Houlbrooke *et al.* (1997) reported that biomass of roots and shoots of ryegrass (*Lolium perenne*) was higher when plants were grown in soil with lower bulk density.

Unlike the soil physical characteristics those influenced comparative clearly on the growth and yield of *H. sempervirens*, the chemical properties affected on plants quite complicatedly. Indeed, it is difficult to explain the effects of individual or combined nutrient element(s) of soils on *H. sempervirens* in this study. According to Fosler and Kamp (1954), without fertilizer application, daylilies grow well in soils that contained high levels of phosphorus (P), and potassium (K), and moderate content of nitrogen (N). However, in this study, although dark-red soil contained moderate content of N (both NO_3-N and NH_4-N) and K, and low content of P, *H.*

sempervirens growing in this soil still grew well and created the highest yield in comparison with gray and red soils. Especially, gray soil contained the highest content of N, P, K, but the growth and yield of plants in this soil were lower than that in dark-red soil (Table 2.1 and 2.2). These results may be explained by influences of N, P, K amount applied for plants in this experiment. It was assumed that the combination of N, P, K at amount of 0.4, 0.4, 0.4 g/pot, respectively with the constituents and content of other chemical element in dark- red soil was better for *H. sempervirens* growth and creating the yield in comparison to the other soils. And with the components and content of chemical elements in gray soil or red soil, *H. sempervirens* would need supplying other amounts of N, P, K to grow and yield best. It is well known that the unbalanced nutrients result in lower growth and yield of crops. And the inherent nutrient contents and their interaction in dark-red soil were probably appropriate for *H. sempervirens* to grow well and create high yield than those in the two other soils.

Although *H. sempervirens* plants in dark-red soil produced up to 49% and 114% significantly higher leaf yield and number flowers, respectively than ones in red soil, they were not different in accumulation OPT (Table 2.2). It is assumed that the similar contents of some elements in these two soils such as Fe, Cu, Mg, and Al probably promoted the accumulation of OPT of plants in dark-red soil and red soil. And the combination of the high content of, Fe, Cu, Mn, Ca, S, and Na and the low content of Mg and Al in gray soil likely restrained OPT accumulation of *H. sempervirens.* Kirakosyan *et al.* (2003) pointed out that the type and composition of soil are two of many environmental factors which influence the phytochemical content of plants and products derived from them. Reimberg *et al.* (2009) indicated that the content of total flavonoids in leaves of *Passiflora incarnata* plants was negatively correlative to

the levels of Fe, Cu, and B in soil. Overall, of the three soils examined in this study, dark-red soil was advised to use for cultivation of *H. sempervirens* with OPT purpose.

2.3.3. Effects of N, P, K fertilizations on growth, yield and OPT concentration

The variation of growth, yield, and OPT accumulation in leaves of *H. sempervirens* influenced by quantitative combinations of N, P, K fertilizer are presented in Fig. 2.3 and 2.4. In general, the growth and leaf yield of this plant were directly proportional to the applied quantity of N, P, K. Treatments of F1–4, receiving the greatest quantities of nutrients, especially nitrogen (0.4 – 0.6 g/pot), resulted in the better growth parameters and higher leaf yield of the plants growing in these treatments as compared to treatments of F5-8, getting 0.0 – 0.4 g/pot of N, P, K fertilizer (Fig. 2.3 and 2.4A). Specifically, the plant height of plants supplied N, P, K as in treatments of F1–3 was significantly higher than that in F5–7, whereas that in F4 was ambiguous (Fig. 2.3A). Treatments of F1 and F2 produced the most tillers with 3.9 tillers/pot, slight higher than number of tillers produced by treatments of F3 and F4 (3.3 tillers/pot), but the difference was not significant. The number of tillers propagated in F5–7 was the lowest in with 2.4 – 2.6 tillers/pot, except that in F8, creating 1.3 tillers/pot (Fig. 2.3B). The clearly difference about number of leaves was observed between group of F1–4 treatments, creating 6.6 – 7.0 leaves/plant, and group of F5–7, producing 5.8 – 5.9 leaves/plant. The number of leaves gave by F8 was the lowest (Fig. 2.3). The quantitative combinations of N, P, K as in treatments of F2–3 resulted in the highest leaf biomass of *H. sempervirens*. These results were statistically higher than those in F6–7, but not significantly higher than those in the other combinations (Fig. 2.3D). The treatment of F8, no fertilizer application, showed the lowest values for all measured parameters of growth, yield, as well as OPT concentration (Fig. 2.3 and 2.4).

Similarly, the leaf yield of *H. sempervirens* in F1–4 was 21.2 – 26.6 g/pot, significantly higher than that in F5–7, ranging 14.6 – 16.6 g/pot. This value in F8 was the clearly lowest with 5.2 g/pot. Among treatments of F1–4, F1 and F2 gave the significantly higher yield in comparison with F4. The treatment of F3 was ambiguous (Fig. 2.4A).

Different from the growth and leaf yield, the flower yield and OPT accumulation of *H. sempervirens* were not proportional to the applied quantity of N, P, K (Fig. 2.4B, 2.4C and 2.4D). Although plants in F1–3 were supplied the most fertilizer (0.4 – 0.6 g/pot), they produced only 3.8 – 3.9 flowers/pot, equivalent to number of flowers produced by plants in F7, supplied N, P, K at level of only 0.2 – 0.3 g/pot. And the number of flowers in these treatments was significantly less than that in F4, applying N, P, K at 0.4, 0.4, 0.4 g/pot and producing 8.2 flowers/pot. The N, P, K combinations as in F5–6 also provided quite high number of flowers (6.0 – 6.4 flowers/pot), but they were not significantly different from the other combinations, including treatment of F8, which did not produce any flowers (Fig. 2.4B). The weight of flowers fluctuated slightly with the N, P, K fertilizations. It ranged from 2.8 to 3.1 g/flower. And only one significantly difference in weight was observed between flowers in F4–5, reaching 3.0 – 3.1 g/flower, and flowers in F7, weighting 2.9 g/flower (Fig. 2.4C). Among treatments applying fertilizer, the fertilization as in F5 produced the significantly highest concentrations of OPT with 2.8 mg/g, next by F6 with 2.5 mg/g. Plants growing in the other fertilizations (F1–4 and F7) accumulated OPT at levels of 2.1 – 2.4 mg/g. The non-fertilizer treatment also produced a decent concentration of OPT (1.9 mg/g), significantly lower than OPT amount of all the fertilizer treatments.

On the whole, it is not easy to conclude which N, P, K fertilization is better for all growth, yield and OPT accumulation of *H. sempervirens* cultivated in dark-red soil. The treatments of

F1–3 promoted the better growth and leaf yield, F4–6, especially F4 were the better choice for flower yield, and F5–6 were ideal for OPT accumulation (Fig. 2.3 and 2.4). However, effectively, the N, P, K application as F4 treatment accumulated only 2.1 mg OPT per g of fresh leaves, equivalent to 79.2% and 90.2% of the average OPT concentration accumulated in F5–6 (2.7 mg/g) and in F1–3 (2.3 mg/g), respectively. And the leaf yield in F4 was 21.2 g/pot, equivalent to 84.5 and 135.4% of the average leaf yield in F1–3 (25.1 g/pot) and in F5–6 (15.7 g/pot), respectively. But the number of flowers produced in F4 was 8.2 flowers/pot, equivalent to 211.9% and 132.3% of the average number of flowers in F1–3 and F5–6, respectively. On the other hand, the weight of flowers in all these treatments was not different. And quantity of OPT in flowers was 3.2 mg/g, 55.3% higher than that in leaves (2.0 mg/g). Although the average leaf yield in F1–3 was 25.1g/pot, about 60% over that in F5-6, the number of flowers and OPT concentration were about 38% and 12%, respectively less than those in F5–6. While the average amount of N, P, K applying in F1–3 was 0.6, 0.5, 0.5 g/pot respectively, about 40% higher than amount of N, P, K used in F5–6 (0.35, 0.3, 0.3 g/pot). Consequently, for cultivation *H. sempervirens* in dark-red soil with OPT purpose, the combination of N, P, K as in F4 is strong recommended, followed by F5–6.

Chaturvedi *et al.* (1988) reported that N had significantly influences flower production of daylily, but P did not. And his study showed that N level at 20g per a bed of size 90 x 90 cm, (equivalent to approx. 246.9 kg/ha or 0.5 g/pot) gave the greatest number of flowers/stem. Similarly, Pandey *et al.* (1993) pointed out that different from P, N affected clearly on the flower yield of *H. fulva,* and the optimum level of N and P for flower yield of this plant was 185.1 and 164.6 kg/ha (equivalent to 0.4 and 0.3 g/pot), respectively. The fertilizers in general and N, P, K

in particular is well known for their influence on metabolism and biomass production. Our study showed that the more N that was supplied with high levels of P and K, the better growth and the higher leaf yield of *H. sempervirens*, but they did not promote the flower production and OPT accumulation. These results are in agreement with the study of Anwar *et al.* (2010), demonstrating that the fresh herb yield of all six cultivars of methol mint (*Mentha arvensis*) reached the highest at the high supplied NPK level (200, 80, 80 kg/ha N, P, K, respectively), but their oil yield was the highest at N, P, K level of 150, 60, 60 kg/ha, respectively. Similarly, Oloyede (2012) reported that of four N, P, K levels (0, 90, 180, and 270 kg/ha) supplying for pumpkin leaf vegetable plants (*Cucurbita pepo*), the levels of N, P, K at 180 and 270 kg/ha promoted the best growth and produced the highest yield, and the level at 180 kg/ha is optimum for accumulation of some antioxidant compounds, such as phenolic, flavonoids, anthocyanin, and proanthocyanin in leaves of this plant.

It is known that N, P, and K are the three essential macro-nutrients of plants. Each factor plays a certain role in the metabolism and yield of plants, such as N is responsible for strong stems and foliage. This element has a decisive role, improving the yields of many crops up to 26 – 41% (Maier *et al.*, 1994, 1996); K improves overall health and disease resistance. The sufficient supply of K promotes efficient N uptake of plants; and P aids in healthy root growth and flower and seed production. It indirectly promotes plant growth and absorption of K as well as other nutrients (Oya, 1972, Akamine, 2007). But they together support growth, yield and quality of plants (Mazid, 1993, Ivonyi *et al.*, 1997). Each particular crop type requires a particular ratio of N, P, and K fertilizers for its optimum growth and development. The balanced utilization of N, P, and K increases growth, yield and quality of crop. The role of each element in

the growth, yield, and OPT concentration of *H. sempervirens* was not examined by this study and requires further elucidation.

Table 2.1 Chemical and physical properties of dark-red soil, gray soil, and red soil in Okinawa, Japan

Properties	Soil types		
	Dark-red soil	Gray soil	Red soil
NO_3-N (mg/kg soil)	12.93	36.82	12.07
NH_4-N (mg/kg soil)	23.83	19.46	14.73
P (mg/kg soil)	4.81	9.59	3.79
K (mg/kg soil)	27.16	50.14	11.75
Ca (mg/kg soil)	133.22	543.28	362.44
Mg (mg/kg soil)	20.99	18.36	29.01
Na (mg/kg soil)	62.18	76.27	57.91
Al (mg/kg soil)	45.38	39.20	41.34
Fe (mg/kg soil)	44.52	269.19	79.33
Cu (mg/kg soil)	0.57	4.71	1.10
S (mg/kg soil)	85.22	276.53	191.33
Zn (mg/kg soil)	3.86	21.51	21.86
B (mg/kg soil)	2.59	3.08	6.38
Mo (mg/kg soil)	4.14	3.24	3.47
Mn (mg/kg soil)	10.66	13.72	2.40
C (%)	0.38	1.44	0.76
pH (2.5 H_2O)	6.4	7.3	7.3
Bulk density (g/cm^3)	0.81	0.89	0.92
Clay (%)	59.3	40.7	28.7
Silt (%)	28.3	27.5	26.8
Sand (%)	12.4	31.8	44.5

Notes: Data were recorded on dry-weight basis. Data are means of three replications.

Table 2.2 Influences of three soil types in Okinawa on growth, yield, and OPT concentration in leaves of *H. sempervirens*

Soil types	Plant height (cm)	Number of tillers (No./pot)	Number of leaves (No./plant)	Leaf biomass (g/plant)	Leaf yield (g/pot[1])	Flower yield		OPT concentration in leaves (mg/g)
						Number of flowers (No./pot)	Flower weight (g/flower)	
Dark-red	26.5 ± 0.5 a	3.8 ± 0.1 a	7.7 ± 0.2 a	6.9 ± 0.2 a	25.7 ± 0.5 a	8.7 ± 1.4 a	3.1 ± 0.04 a	2.2 ± 0.01 a
Gray	24.5 ± 0.4 b	3.2 ± 0.2 b	6.5 ± 0.1 b	6.2 ± 0.4 ab	18.5 ± 1.7 b	5.7 ± 1.4 ab	3.0 ± 0.04 ab	1.9 ± 0.01 b
Red	22.9 ± 0.4 c	3.3 ± 0.2 b	6.3 ± 0.1 b	5.5 ± 0.3 b	17.2 ± 1.2 b	4.1 ± 1.3 b	2.9 ± 0.05 b	2.2 ± 0.01 a

Notes: Data are means ±SE (standard errors) (n= 30), except parameter of OPT concentration in leaves (n=3). Mean values in each column followed by the same lowercase letters are not significantly different (P<0.05) by LSD.

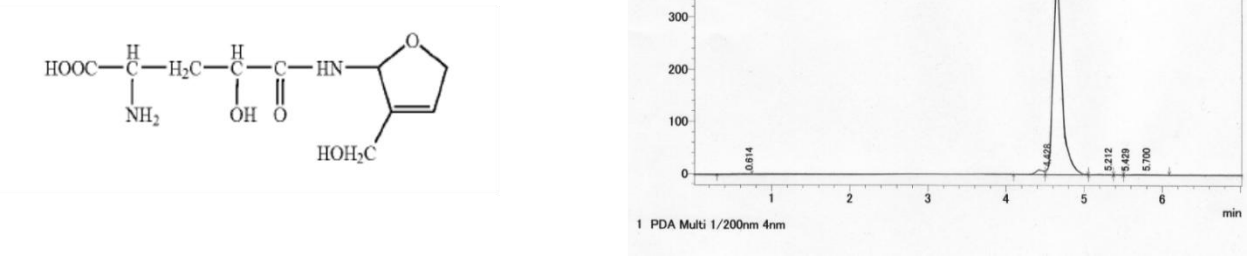

Fig. 2.1 Chemical structure and HPLC chromatograph of Oxypinnatanine.

Note: Chemical structure of OPT is cited from Ogawa *et al.*, 2013.

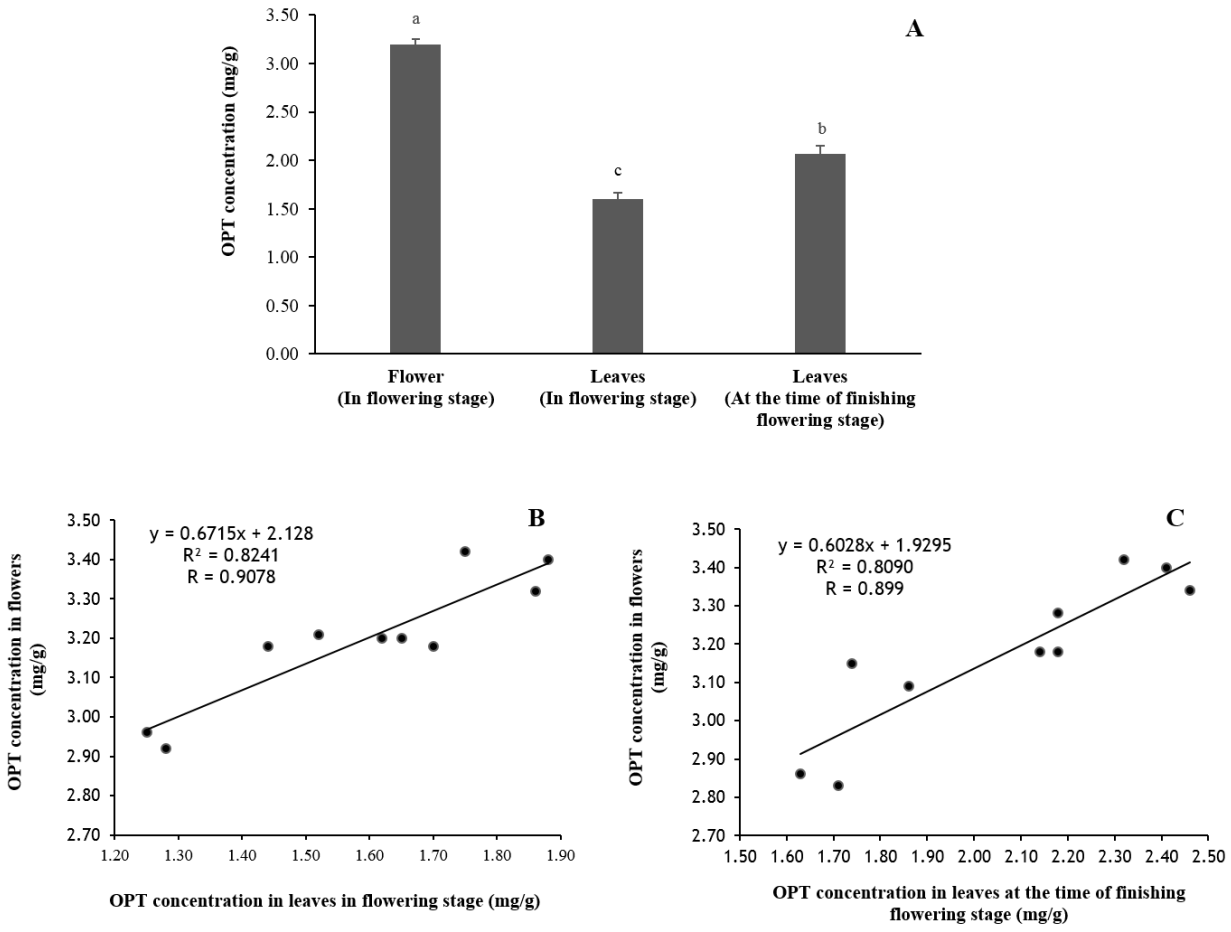

Fig. 2.2 Relationship between the OPT concentration in the flowers and leaves of *H. sempervirens*.

Notes: A: The OPT concentration in the flowers, leaves in flowering stage, and leaves at the time of finishing flowering stage. B: Correlation between the OPT concentration in the flowers and leaves in the flowering stage. C: Correlation between the OPT concentration in the flowers and leaves at the time of finishing flowering stage. Bar on each column represents SE (standard errors) of the mean (n=10). Data in columns with the same lowercase letters are not significantly different ($P< 0.05$) by LSD.

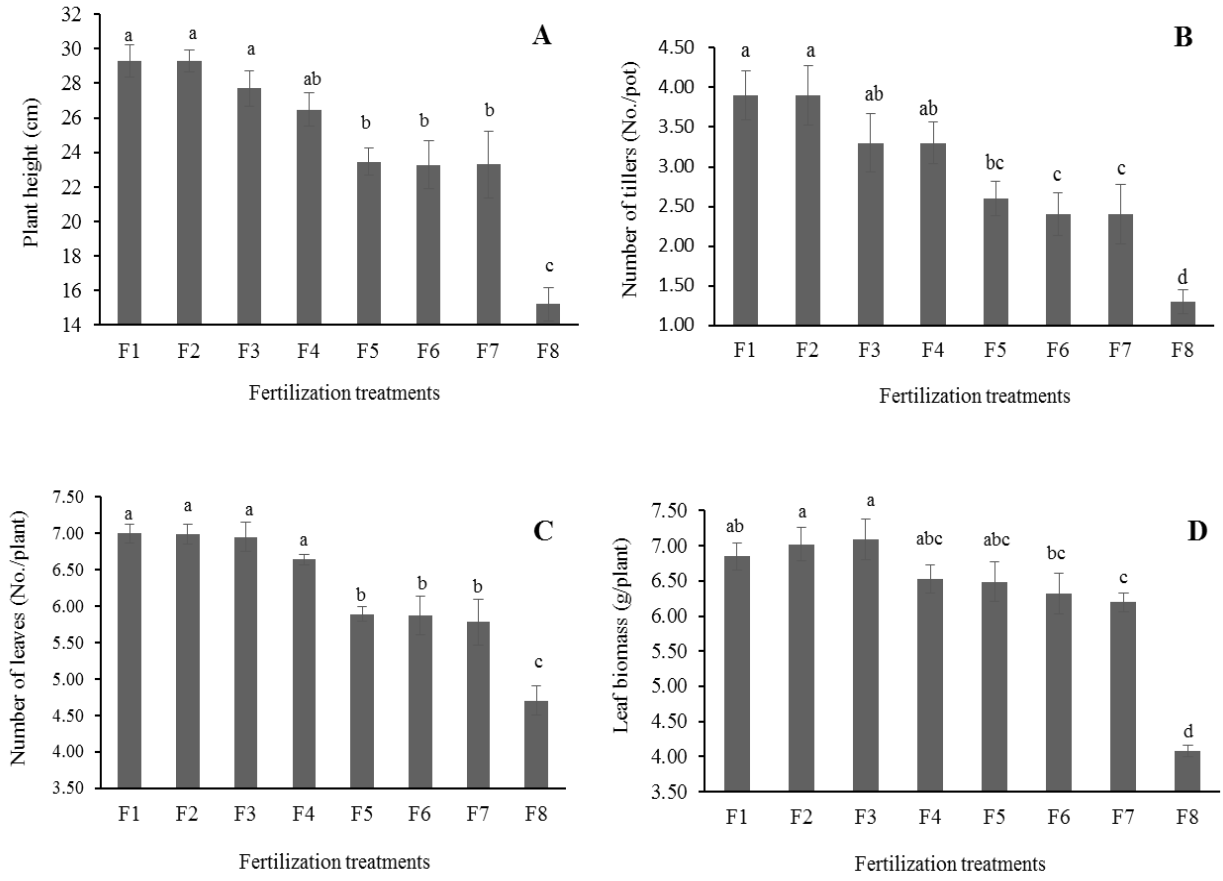

Fig. 2.3 Effects of N, P, K fertilizations on plant height *(*A), number of tillers (B), number of leaves (C), and leaf biomass (D) of *H. sempervirens.*

Notes: Bar on each column represents SE (standard errors) of the mean (n=10). Data in columns with the same lowercase letters are not significantly different ($P< 0.05$) by LSD. Fertilization treatments of N, P, K (g/pot N: P_2O_5: K2O, respectively):

F1. 0.6: 0.6: 0.6 F2. 0.6: 0.5: 0.5 F3. 0.6: 0.4: 0.4 F4. 0.4: 0.4: 0.4

F5. 0.4: 0.3: 0.3 F6. 0.3: 0.3: 0.3 F7. 0.3: 0.2: 0.2 F8. 0: 0: 0

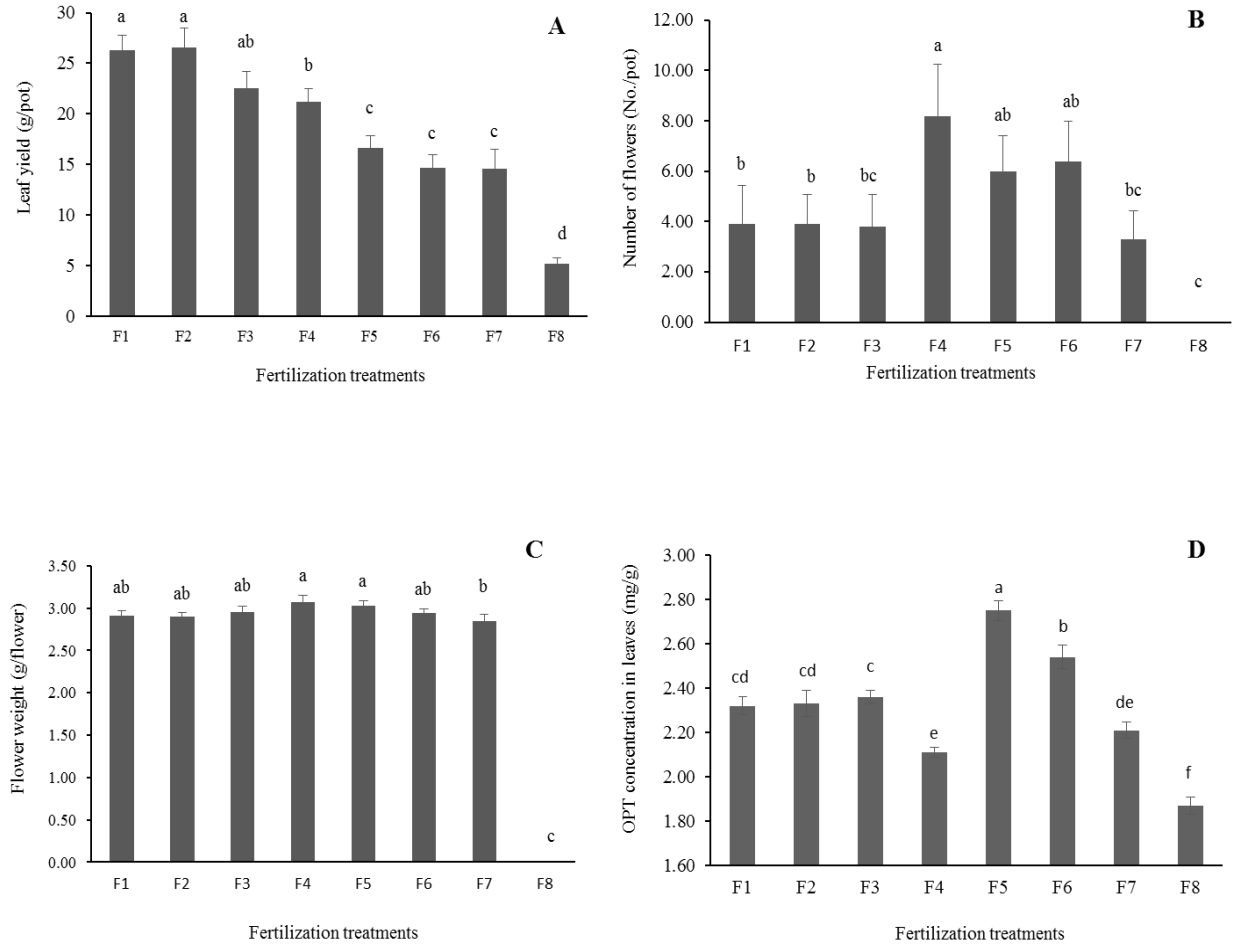

Fig. 2.4 Effects of N, P, K fertilizations on leaf yield (A), flower yield (B and C), and OPT concentration in leaves (D) of *H. sempervirens*.

Notes: Bar on each column represents SE (standard errors) of the mean (n=10) (n=3 for columns in Fig. 2.4 D). Data in columns with the same lowercase letters are not significantly different ($P< 0.05$) by LSD. Fertilization treatments of N, P, K (g/pot N: P_2O_5: K2O, respectively):

F1. 0.6: 0.6: 0.6 F2. 0.6: 0.5: 0.5 F3. 0.6: 0.4: 0.4 F4. 0.4: 0.4: 0.4

F5. 0.4: 0.3: 0.3 F6. 0.3: 0.3: 0.3 F7. 0.3: 0.2: 0.2 F8. 0: 0: 0

CHAPTER 3

Effects of Some Environmental Factors on Oxypinnatanine Concentration in Leaves of *Hemerocallis fulva* var. *sempervirens* in Okinawa, Japan

3.1. Introduction

Okinawa is an island group located at the southern end of Japan. This region has a subtropical climate, creating the very diverse floral. The inhabitants of Okinawa are frequently cited as the longest life span people in the world. They have traditionally utilized the herbs and edible plants as foods and medicines that contribute to their longevity. *H. sempervirens* growing popularly in Okinawa has been used commonly to control insomnia by local people. The sedative and sleep-improving effects of *H. sempervirnes* have attracted attention of many researchers. Some recent studies have found that oxypinnatanine (OPT) (shown in Fig. 2.1), an amino acid amide and accumulated by *H. sempervirens* is an active compound for the sedative and sleep-improving effects (Urade *et al*., 2010, Ogawa *et al.*, 2013). However, although there are many studies on active mechanisms and sleep-aiding effects of OPT isolated from *H. sempervirens*, there are no investigation of OPT accumulation as well as fluctuation of OPT concentration in plants as influenced by cultivation and environmental conditions.

Insomnia is a widespread health complaint, and the most common of all sleep disorders (Morin *et al*., 1999). Approximately 35% of the adult population has insomnia during the course

of a year. Up to 7% show insomnia is a chronic, severe, or both (Ford and Kamerow, 1989, Nowell *et al.*, 1997, Johnsn *et al.*, 1998). In contrast to the occasional sleepless night undergone by most people, insomnia may be a persistent or recurrent problem with serious complications such as anxiety and depression (Kales *et al.*, 1984, Ford and Kamerow, 1989, Mendelson, 1995). Conventional medical treatment for insomnia includes psychological and pharmacological approaches. However, long-term use of frequently prescribed medications can lead to habituation and problematic withdrawal symptoms (Anoja *et al.*, 2000). The presently used sleeping pills have reported to induce side effects such as benzodiazepines cause drowsiness, anterograde amnesia and dependence (Anoja *et al.*, 2000, www.webmd.com), antidepressants bring about anticholinergic effects, Z-drugs (Ambien, lunesta and sonata) can also cause some problems such as day time confusion, memory issues and falls (Adams, 2014). Therefore, herbal and other natural sleep aids are gaining popularity, as herbs commonly used for their sedative-hypnotic effects do not have the drawbacks of conventional drugs.

According to many studies for improving the quantity of bioactive compounds in medicinal plants, the accumulation and preservation of pharmaceutical active compounds in plants are influenced by numerous factors from crop cultivation to extraction and storage of raw materials, such as cultivation techniques, internal factors of plants, environmental conditions, and store conditions (Ciccarelli *et al.*, 2001, Bottcher *et al.*, 2003, Schilter *et al.*, 2003, Poutaraud and Girardin, 2005). Thus, the precise determination of effects of these environmental factors is considered as the first step toward the control and optimization of the biosynthesis and accumulation of specific secondary metabolites in herbaceous crops to improve the quality of drug production (Poutaraud and Girardin, 2005). It is thought that, same as the other secondary

metabolites of medicinal plants, the biosynthesis and accumulation of OPT of daylily are influenced substantially by many factors. And present study was carried out to evaluate influences of some environmental factors such as waterlogged soil, temperature, light intensity, harvesting time, and the post-harvest storage methods to OPT concentration in leaves of *H. sempervirens* in Okinawa in order to control the OPT amount in this plants and preserve that in raw materials.

3.2. Materials and Methods

3.2.1. Plant materials

The *H. sempervirens* plants, preserved in the experimental region of Faculty of Agriculture of University of the Ryukyus, Okinawa, Japan (26º21'N - 127º48' E) were used as the plant material for present study.

3.2.2. Plant cultivation for experiments of effects of waterlogged soil, relative light intensity, and temperature on OPT concentration in *H. sempervirens* leaves.

Experiments for evaluation of influences of waterlogged soil, temperature, and relative light intensity on OPT concentration in *H. sempervirens* leaves were carried out from July 2012 to September 2013. The preserved *H. sempervirens* (described in section 3.2.1) was transplanted into Wagner pot (size 1/5000a), filled with about 3 kg of air-dried soil, one plant per pot. Each treatment consisted of nine pots for nine replications. Then, the potted plants were placed using a completely random design in the net house. Dark-red soil, a common soil in Okinawa was used for these experiments. Fertilizer amount of N: P_2O_5: K_2O utilized for the experiments was 0.4: 0.4: 0.4 g/pot, respectively (equivalent to 200: 200: 200 kg/ha, respectively). The whole dose of P_2O and K_2O, and one-half the amount of nitrogen were applied at the time of planting and the

remaining quantity of nitrogen was applied 6 months after planting. No organic fertilizer was utilized for these experiments.

In all three experiments, plants were harvested three times within August to September 2013: at the early, middle and late stages of the flowering period. Each times, three samples from three pots of each treatment were gathered for the determination of the OPT concentration.

Experiment of effects of waterlogged soil on OPT concentration

After planted in Wagner pots, filled with about 3 kg of air-dried soil, the plants were grown in condition of adequate water as in the common cultivation method for *H. sempervirens* in Okinawa (well-watered soil) and in soil that was always immersed in water (waterlogged soil) throughout process of experiment.

Experiment of effects of relative light intensity (RLI) on OPT concentration

From planting to harvesting, from July 2012 to September 2013, plants were grown using four treatments, including 100% relative light intensity (RLI) (without shade), 75% RLI (covered by one sheet of 3-mm-mesh net), 60% RLI (covered by two sheets of 3-mm-mesh net), and 40% RLI (covered by three sheets of 3-mm-mesh net). Percentage of RLI was calculated by following formular:

$$Relative\ Light\ Intensity\ (RLI)(\%) = \frac{Light\ Intensity\ inside\ net}{Light\ Intensity\ outside\ net} \times 100$$

Experiment of effects of temperature on OPT concentration

Plants for this experiment were grown in the same net house. Before harvesting, during the flowering stage (within August to September 2013), they were exposed for 10 days to the

temperature at 10°C, 15°C, 20°C, 25°C, 30°C, 35°C and 40°C in growth chambers. The light at about 210 µmol/m^2/s and relative humidity at 60% were set up.

3.2.3. Evaluation of effects of harvesting time on OPT concentration in *H. sempervirens* leaves

The leaves of the *H. sempervirens* plants (described in section 3.2.1), were sampled every month, from January to December 2013. Each month, three samples, consisting of whole leaves of 3 plants were harvested for determination of the OPT concentration.

3.2.4. Evaluation of effects of storage methods on variation of OPT concentration in post-harvested *H. sempervirens* leaves

After harvested, *H. sempervirens* leaves were stored by four conditions of room temperature, 0 – 4ºC, –20ºC, and dried leaves. These experiments were conducted from December 2013. The leaves of *H. sempervirens* (described in section 3.2.1) were harvested and divided into many small portions, each was 10 g. Then these portions were placed into store conditions to use for quantitative analysis of OPT. Some portions were dried at 50ºC until the weight was invariable.

The leaves stored at condition of room temperature were analyzed to determine the OPT concentration every day until they turned yellow and rot.

After stored at condition of 0 – 4ºC for 3, 5, 10, 12, 15, 18, 20, 22, 25 days, the leaves analyzed to determine the OPT concentration.

After stored at condition of –20ºC for 30, 45, 60, 90, 120, 150, 180, 210, and 240 days, the leaves analyzed to determine the OPT concentration.

The dried leaves were stored in the plastic bags until the time of analysis (30, 90, 180, and 240 days of store periods).

All analysis were conducted triplications

3.2.5. Determination of OPT concentration

The determination of OPT in *H.sempervirens* leaf samples for experiments in this chapter was conducted as the procedure described in chapter 2, section 2.2.7.

3.2.6. Statistical analysis

Recorded data were statistically analyzed as descriptions in chapter 2, section 2.2.9.

3.3. Results and Discussion

3.3.1. Effects of waterlogged soil, relative light intensity, and temperature on OPT concentration in *H. sempervirens* leaves

The relationships between environmental factors and the amount of OPT in *H. sempervirens*, estimation of OPT concentration of plants cultivated in different conditions including waterlogged soil, shade, and environmental temperature was performed. The results are presented in Fig. 3.1.

Effects of waterlogged soil on OPT concentration

Results of quantitative estimation of OPT concentration in leaves of *H. sempervirens*, cultivated in waterlogged soil and well-watered soil, indicated that the OPT concentration in the plants cultivated in waterlogged soil, 1.64 mg/g was not significantly different from that in the plants cultivated in well-watered soil, 1.84 mg/g (Fig. 3.1A).

Waterlogged soil influences synthesis and accumulation of secondary metabolite in plants by different ways depeding on a specific chemical compound in a particular plant. For example,

waterlogging significantly reduced the content of three alkaloids: berberine, jatrorrhizine and palmatine in amur croktree (*Phellodendron amurense*) seedlings (Xia *et al.*, 2007). Inga *et al.* (2012) examined the effects of water stress on the levels of glucosinolates and several amino acids in phloem sap of *Arabidopsis thaliana* and the results showed that after more than 1 week subjected to water stress, the concentrations of glucosinolates and amino acids such as glutamic acid, aspartic acid, proline, and isoleucine in the phloem sap of waterlogged plants were statistically lower than those of drought-stressed plants, and non-significantly lower than those of well-watered plants. And in this study, waterlogging did not affect OPT accumulation of *H. sempervirens.* However, the evaluation of effects of waterlogged soil and well-watered soil on growth and yield of *H. sempervirens* should be conducted futher in order to have the best choice for cultivation of this plant.

Effects of relative light intensity (RLI) on OPT concentration

The relative light intensity affected remarkably the variation of OPT concentration in the leaves of *H. sempervirens*. Under high relative light intensity (100% RLI) and very low RLI (40%), the plants produced OPT at 1.96 and 1.88 mg/g, respectively. These amounts were significantly higher than those of plants grown under 75% and 60% RLI, 1.22 and 1.38 mg/g, respectively (Fig. 3.1B).

The quantity and quality of light influence the secondary metabolism of plants, either directly by the accumulation of energy/carbohydrates, or indirectly by regulating metabolic pathways (Poutaraud and Girardin, 2005). The level of hypericin in leaves of *Hypericum perforatum* increased continuously along with the increase in light intensity, and this was thought to be linked to an increase in the number of dark glands (Briskin and Gawienowski, 2001).

Moderate light intensity, 301−600 lux, improved the anthocyanin content in *Melastomama labathricum* (Chan *et al*., 2010). With regard to *H. sempervirens* cultivated in Okinawa, conditions of 100% and 40% RLI were better for accumulating OPT in leaves in comparison with conditions of 75% and 60% RLI. But condition of 40% RLI maybe affect photosynthensis efficacy, influencing directly on creating yield of plants.

Effects of temperature on OPT concentration

After 10 days of growth under different temperature conditions, the plants subjected to high temperature (40°C) produced the significantly highest level of OPT, 3.57 mg/g, followed by the plants exposed to low temperature (15°C), 2.92 mg/g. The lowest concentration of OPT, 0.48 mg/g, was found in the plants treated at 10°C. There were no significant differences in the OPT accumulation in plants treated at 20 − 35°C; the concentrations ranged from 1.72 to 1.89 mg/g, which was significantly lower than the concentrations achieved at 40°C and 15°C (Fig. 3.1C).

Like the light intensity, the ambient temperature is a crucial environment factor, relating strongly to the phenological development and secondary metabolism of medicinal plants (Zobayed *et al*., 2005). At both high and low temperature levels, plants increase their secondary metabolite concentration to maximize their tolerance (Janska *et al.*, 2010). Cold stress increased phenolic compounds which were subsequently incorporated into the cell wall as either suberin or lignin (Griffith and Yaish, 2004). The accumulation of ginsenoside in the herb *Pinax quinquefolius* was enhanced by a 5°C increase in temperature (Jochum *et al*., 2007). In *Hypericum perforatum* along with an increase in the temperature ranged from 24°C to 32°C and an increase in the light intensity ranged from 803.4 to 1618.4 µmol/m^2/ s, increases were detected in the content of hyperforin, hypericin and pseudohypericin (Mehmet *et al.*, 2009; Radušienė *et*

al., 2012). In present reaserch, the OPT concentration in leaves of *H. sempervirens* reached the highest if the plants was treated at 40°C and next by at 15°C.

Secondary metabolites of plants are thought to play a fundamental role in the plants' adaption to the environment and in overcoming stress conditions (Kliebenstein, 2004). For example, phytoalexins and/or phytoanticipins contribute to the protection of plants from pathogens, and several UV- absorbing compounds are accumulated to prevent the serious leaf damage from light. Thus, environmental factors such as the soil condition, water availability, light intensity, and temperature etc. markedly influence the phytochemical contents of plants and the products derived from them (Kirakosyan *et al.*, 2003). These compounds are often accumulated or improved in plants that are undergoing stresses including various elicitors (Ramakrishna and Ravishankar, 2011). To our knowledge there is no published information regarding how OPT affects the function of plants. And our present findings revealed that OPT was synthesized and accumulated the most at conditions of high or low light intensity and temperature. The OPT concentration in leaves of *H. sempervirens* was not affected by waterlogged soil.

3.3.2. Effects of harvesting time on OPT concentration in *H. sempervirens* leaves

The fluctuation of OPT concentration in fresh *H. sempervirens* leaves according to corresponding harvesting time presented in Fig. 3.2 showed that within 1 year, *H. sempervirens* leaves harvested in different time contained different OPT amount. The most OPT accumulated in leaves in December (2.22 mg/g FL) and January (2.15 mg/g), next by in August (1.68 mg/g) and September (1.73 mg/g), the lowest OPT amount was observed in *H. sempervirens* leaves gathered in June (0.45 mg/g) and July (0.63 mg/g) (Fig. 3.2).

Okinawa located at 26º21'N – 127º48' E is a subtropical climate region. Monthly average temperature is 16 − 28.3ºC; rainfall is about 120 – 260 mm; relative humidity is 69 − 85%; sunlight hours is 3.4 − 9.9 h/day; and day length is 10.5 − 13.7 h/day (Fig. 3.3). It is assumed that the interaction of climate parameter in each month influenced accumulation OPT on leaves of *H. empervirens* plants. December and January are the coldest months in Okinawa. The average temperature of these two months is 18ºC and 16ºC, respectively. During this time, some daylily strains undergoes dormant period, but *H. sempervirens* belongs to evergreen daylily group, which retains their leaves throughout the year. It is assumed that OPT plays a defense role to protect plants from unfavorable conditions. Consequently, the combination of low temperature with low relative humidity (69%), low rainfall (approx. 130 mm), short daylength (10.5 – 10.7 h/day), and the low sunlight hour (3.5 − 4 h/day) in December and January in Okinawa was thought to be favorable condition for OPT production of *H. sempervirens*. Similarly, the combination of weather parameters in August and September such as high temperature and relative humidity, 7 – 8 sunlight hours a day, and 12.3 − 13.0 h/day of day-length was assumed to be a good for the OPT accumulation. On the other hand, the flower of *H. sempervirens* in Okinawa starts blooming in August, and flourishes in September, this is also thought to be one of the reasons increasing OPT content in leaves of *H. sempervirens.* The effects of growth stage of daylily on OPT concentration in leaves are mentioned in the next chapter.

As we known, phytochemicals appear to protect plant from unfavorable factors such as herbivore, insects, pathogens, UV ray, and other environmental factors. A particular compound is synthesized in a particular plant and its content in plant depends on many factors such as genotypes, age of plant, growth stages, temperature, light intensity, the climate etc. Up to now,

no report regarding variation of OPT in daylily have been published, but there are many studies demonstrating the variation of other bioactive compounds in other plants caused by climate or seasonal factors. For examples, many studies show that in spring having in intermediate temperature, high light intensity, longer days and dry conditions the brassicaceous plants often accumulate the most total glucosinate concentration (Rosa *et al.*, 1996, Sarwar and Kirkegaard, 1998, Ciska *et al.*, 2000, Rosa and Rodrigues, 2001, Vallejo *et al.*, 2003, Charron and Sams, 2004, Zhang *et al.*, 2006, 2008, Ito and Kimura, Padilla *et al.*, 2007). Rosa and Rodrigues (2001) reported that in some broccoli cultivars, the total and individual glucosinolate concentrations were higher in late season crop in comparison to the early season. Mercadante and Rodriguez-Amaya (1991) presented that content of total carotenoid, β-carotene and combined lutein–violaxanthin in kale cultivar Manteiga growing in winter was higher than that in plants growing in summer, and the carotenoid neoxanthin content in summer grown kale cv. Tronchuda plants was higher than that in winter grown plants. Müller-Riebau *et al.*, 1997 showed that the concentration of the fungitoxic components in the essential oils and the content of carvacrol and thymol constituents of the phenolics *Thymbra spicata* and *Satureja thymbra*, growing wild in the East Mediterranean region of Turkey, were low in the early vegetative stage and increased gradually with plant development. The maximum concentration was reached in June or July, shortly after flowering.

3.3.3. Effects of storage methods on variation of OPT concentration in post-harvested *H. sempervirens* leaves

Storage at room temperature

The results of qualitative of OPT in leaves stored at room temperature shown in Fig. 3.4 indicated that OPT concentration in the *H. sempervirens* leaves stored at room temperature for 2 days (2.34 mg/g fresh leaf (FL), fresh leaves were referred to leaves at the time of harvesting) did not vary in comparison to that in fresh leaves (2.37 mg/g FL). After 3days stored, each g fresh leaves contained only 1.89 mg of OPT. From the fourth day of store processing, leaves became withered and rot, and OPT concentration in leaves was decreased clearly. After 9 days at room temperature, the leaves were nearly total rotted, and OPT in these leaves were only 0.27 mg/g FL.

Storage at 0 – 4ºC

At store condition of 0 – 4ºC, OPT amount in leaves was preserved for long time. During 10 days, OPT concentration nearly did not change in comparison with the OPT amount in fresh leaves. After 18 – 25 days stored at this temperature, the OPT amount in leaves was only 0.21 – 1.24 mg/g FL, decrease 49 – 90 % in comparison to OPT content in fresh leaves (2.37 mg/g) (Fig. 3.5).

Storage at –20ºC

The OPT concentration in *H. sempervirens* leaves stored at minus 20ºC was stable so long time. After 240 store days, OPT amount in leaves was still at level of 1.57 mg/g FL, decrease only about 34% in compared to OPT amount in fresh leaves (Fig. 3.6).

Storage of dried *H. sempervirens* leaves

H. sempervirens leaves, dried at 50ºC until unvariable weight, were lost almost OPT. They contained only 0.61 mg/g FL, decrease about 75% compared to OPT in fresh leaves. During storage period, OPT content in dried leaves nearly did not change (Fig. 3.7).

Willis and Stuart (2000) reported that storage for 60 day in the dark at 5ºC did not affect on alkamide content in the roots of *E. purpurea* plant, but caused a 70% decrease in cichoric acid content; and in a reverse experiment, storage of the ground roots of *E. purpurea* in the condition of 20ºC with the light did not decrease the cichoric acid concentrations, but decreased 65% the alkamide concentrations. Total alkamide content in roots of *E. purpurea* stored at 24ºC for 16 week was 3.28 mg/g dry weight (DW), and decreased to 0.66 mg/g DW at 64th week of storage; in the roots stored at 3ºC this contents was 5.09 and 2.82 mg/g DW at 16th and 64th week of storage, respectively (Perry *et al.*, 2000). Perry *et al.* (2000) also reported that –18ºC is the best storage temperature for roots of *E. purpurea* to preserve the alkamide concentration. The concentrations of parthenolide in dried feverfew leaves stored at –15ºC, 6ºC, and 24ºC for 120 days were not different and preserved (Tanko *et al*., 2003).

The main objective of store of medicinal plants is to preserve their quantity as well as quality. This is done indirectly by controlling moisture and air movement and preventing attacking of insects, rodents, and microorganisms. During storage, the major purpose is to reduce metabolic activity, make the medicinal plant become less susceptible to harmful factors. A particularly phytochemical in a particularly medicinal plant has a response to a specific condition of storage. The OPT amount in *H. sempervirens* leaves stored at –20ºC were preserved the best. And it is easy to be lost if the leaves was dried at 50ºC. However, may be if drying temperature would be decrease, the lost OPT amount of leaves during drying period may be limited.

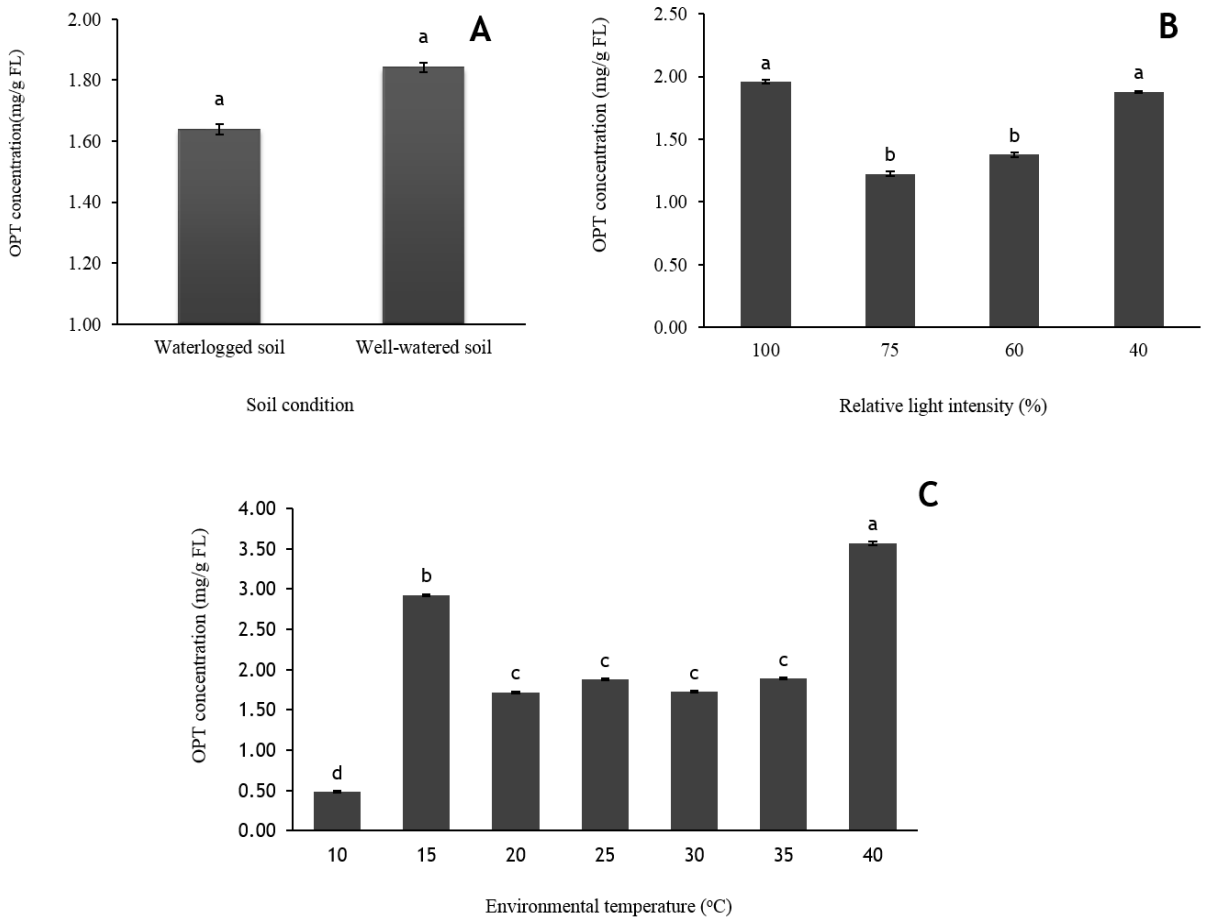

Fig. 3.1 Variation of OPT concentration in *H. sempervirens* leaves influenced by soil condition (A), relative light intensity (B), and environmental temperature (C).

Notes: The vertical bar on each column represents the standard error (SE) value of the mean (n=9). In each chart, columns with the same lowercase letters are not significantly different by LSD ($P<0.05$).

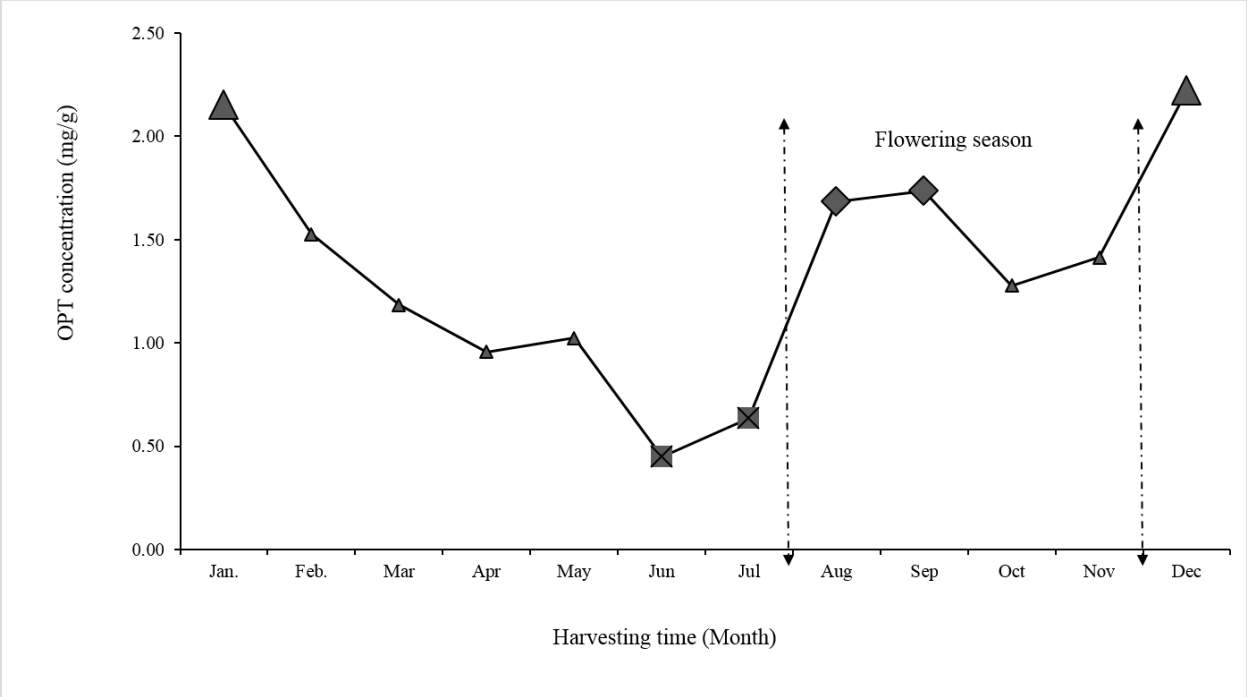

Fig. 3.2 Effects of harvesting time on OPT concentration in *H. sempervirens* leaves.

Notes: Data of each month was the average OPT concentration of 3 samples (n=3).

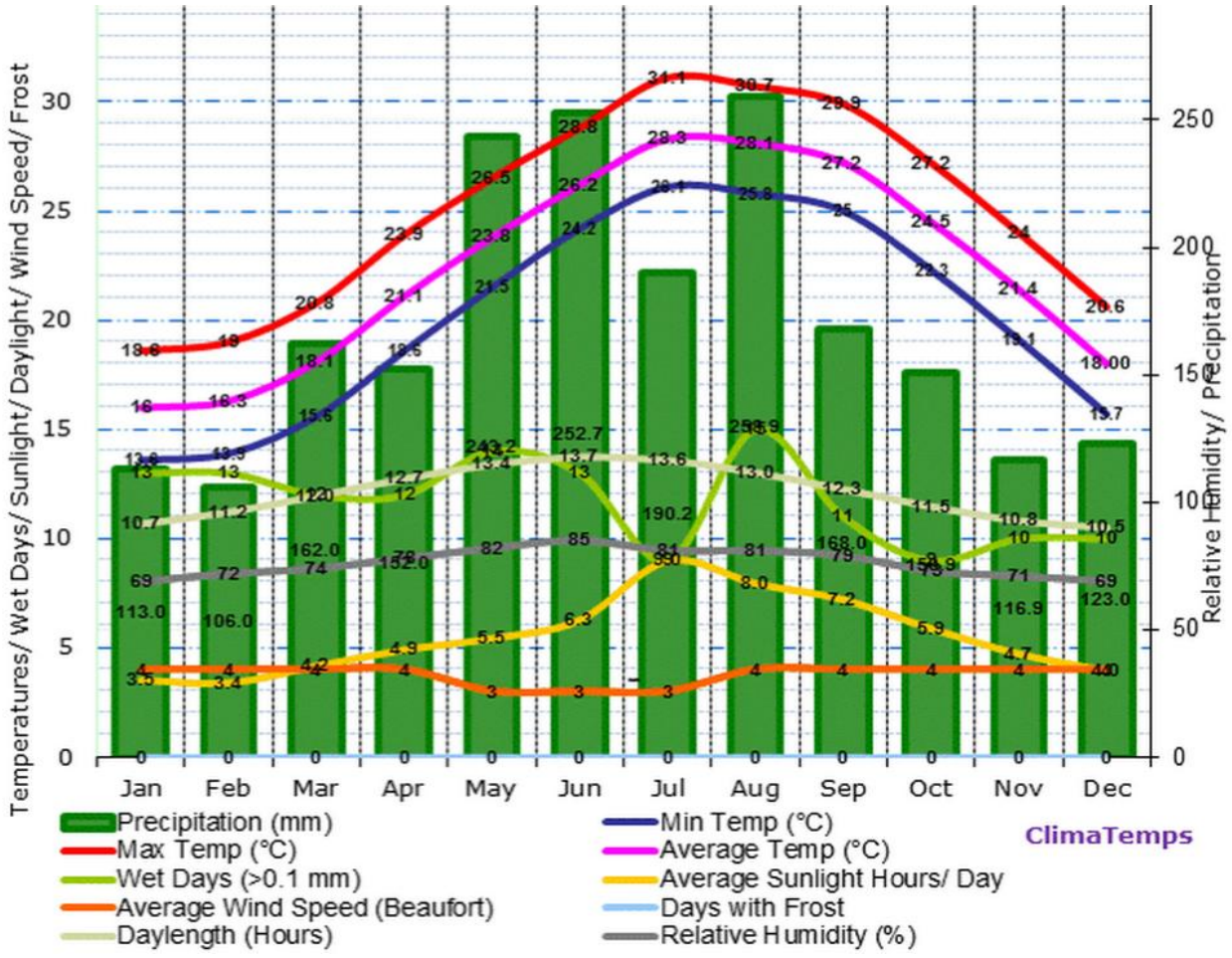

Fig. 3.3 Climate data of Okinawa, Japan.

Note: Source: http://www.naha.climatemps.com/Naha, Okinawa Climate & Temperature

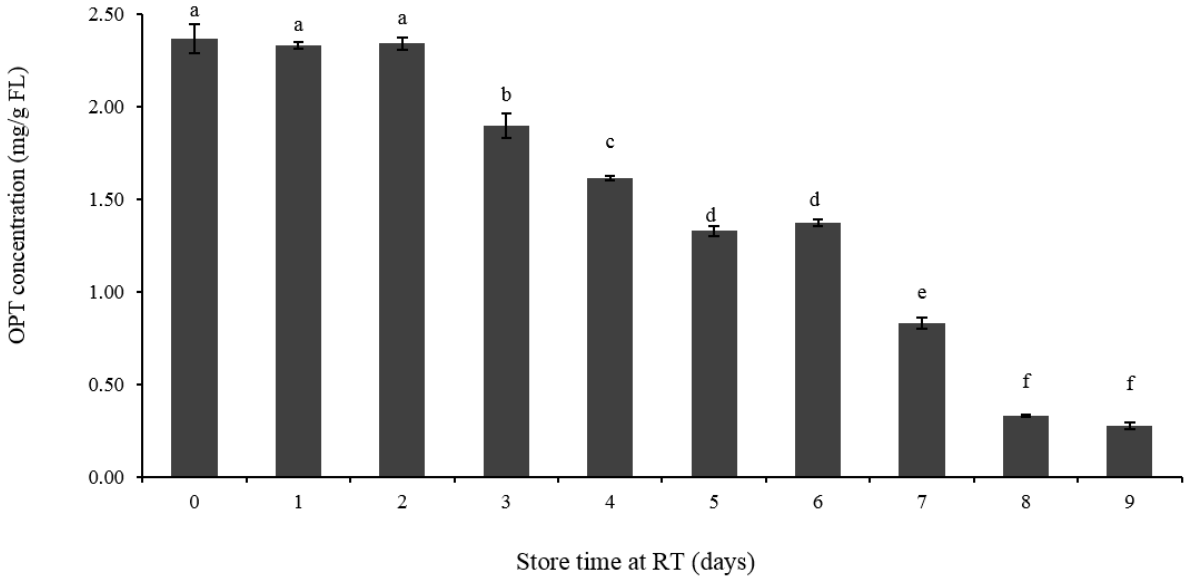

Fig. 3.4 Variation of OPT concentration in *H. sempervirens* leaves stored at room temperature.

Notes: The vertical bar on each column represents the standard error (SE) value of the mean (n=3). In each chart, columns with the same lowercase letters are not significantly different by LSD (P<0.05).

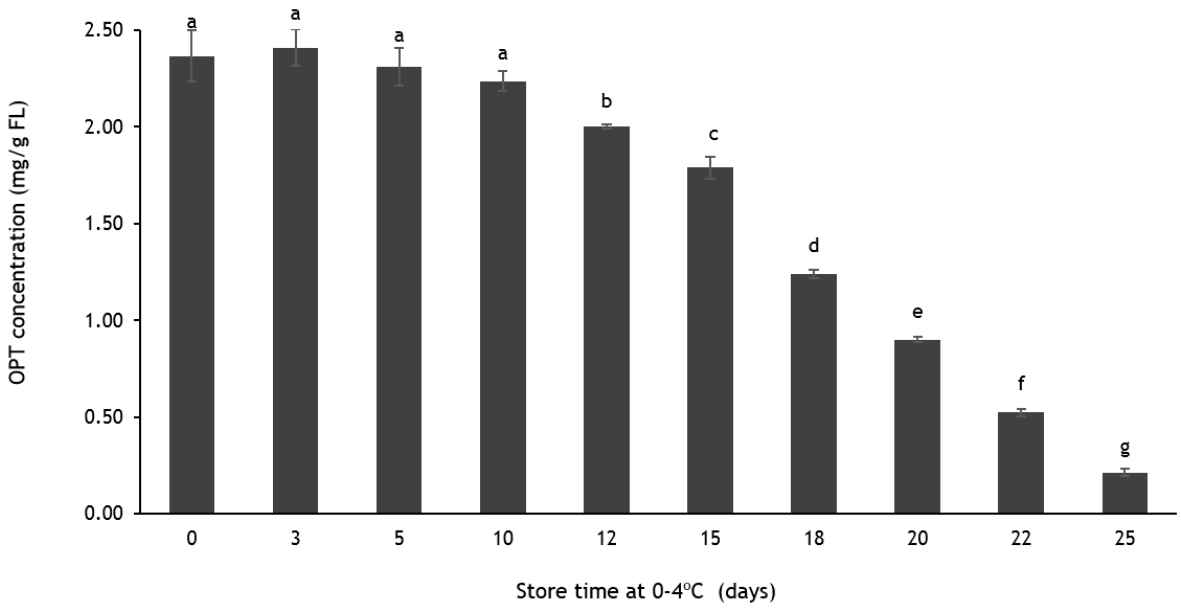

Fig. 3.5 Variation of OPT concentration in *H. sempervirens* leaves stored at 0-4ºC.

Notes: The vertical bar on each column represents the standard error (SE) value of the mean (n=3). In each chart, columns with the same lowercase letters are not significantly different by LSD (P<0.05).

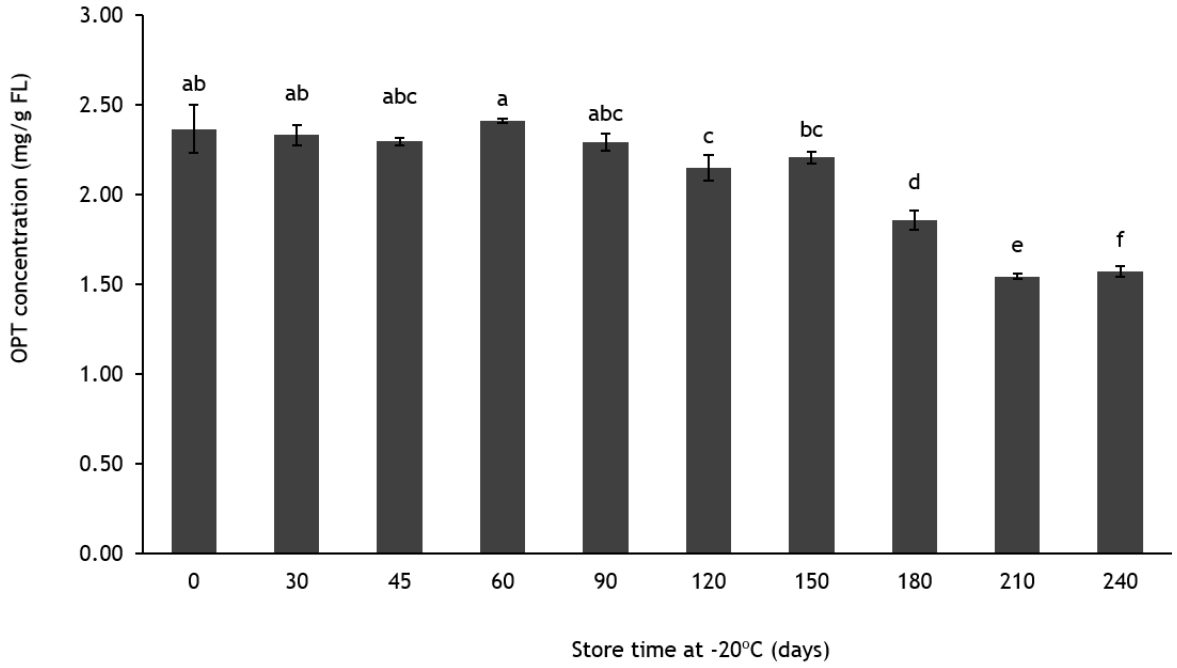

Fig. 3.6 Variation of OPT concentration in *H. sempervirens* leaves stored at - 20ºC.

Notes: The vertical bar on each column represents the standard error (SE) value of the mean (n=3). In each chart, columns with the same lowercase letters are not significantly different by LSD ($P<0.05$).

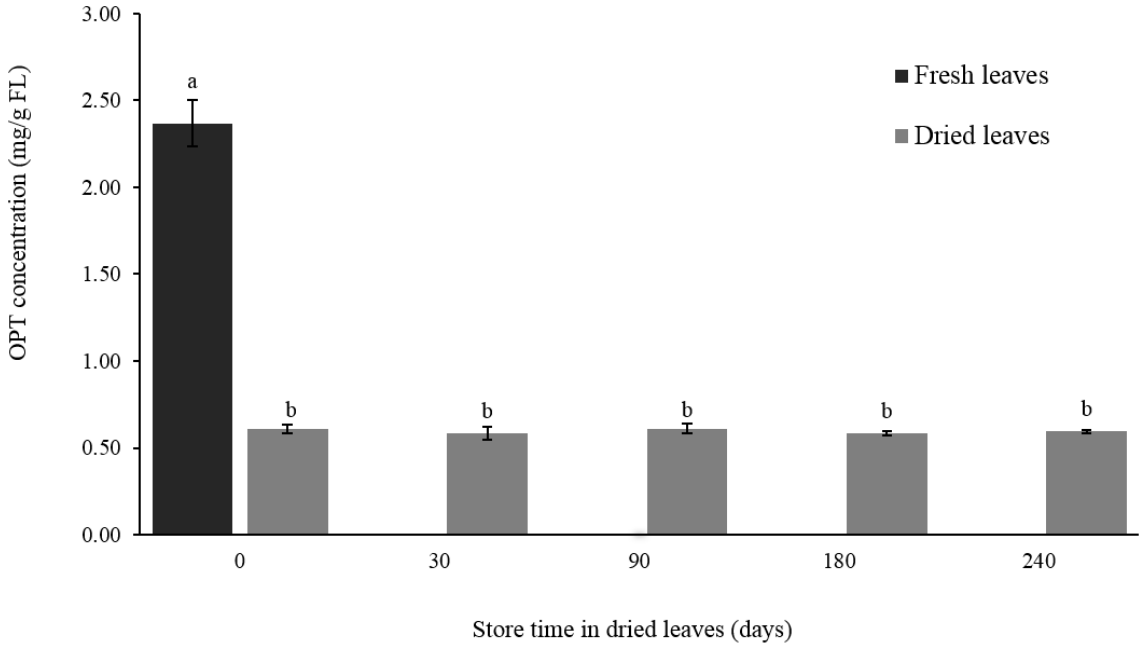

Fig. 3.7 Variation of OPT concentration in dried *H. sempervirens* leaves.

Notes: The vertical bar on each column represents the standard error (SE) value of the mean (n=3). In each chart, columns with the same lowercase letters are not significantly different by LSD (P<0.05).

CHAPTER 4

Relationship between Ploidy Level, Genetic Diversity, and Growth Stage and Variation of Oxypinnatanine Concentration in Leaves of Daylily (*Hemerocallis* spp.)

4.1. Introduction

The ploidy level of daylily (*Hemerocallis* spp. *Hemerocallidaceae*) is variation. It may occur as diploid (2x), triploids (3x) and tetraploid (4x) (Saito *et al*., 2003, Zhang *et al.*, 2013). The basic chromosome number shown by daylily karyotypes is 11 (Stout, 1934, Brennan, 1992). Genetic diversity of daylily is high (Kang *et al.*, 1998, Kang and Chung, 2000, Tomkins *et al.*, 2001, Noguchi *et al.*, 2004, Cao *et al*., 2013). According to Zomlefer (1999), the morphological characters of *Hemerocallis* vary even within a species. So it is difficult to define specific boundaries between species. Daylily cultivars are very diverse in flower color and form. Cao *et al.* (2013) reported that 21 accessions of *Hemerocallis*, collected from ten counties along the Taihang Mountain range, could be clearly distinguished by morphological characteristics. And genetic diversity of these 21 genotypes is so high with 95% polymorphic loci (Cao *et al.*, 2013). Kang and Chung (2000) demonstrated the high level of allozyme variation within 30 populations of five *Hemerocallis* species from Korea and low level of allozyme divergence within species.

Now, there are 19 species of daylily recognized (The World Checklist of Selected of Plant Families, 2014) and more than 60,000 registered daylily cultivars

(https://en.wikipedia.org/wiki/Daylily). However, almost studies on the sedative and sleep-aiding effects of both crude extract and OPT isolated from daylily have been performed by using species *H. fulva*, especially variety *H. sempervirens*. Until now, there are no information, regarding the presence as well as content of OPT in other daylily species or cultivars and influences of internal factors of plants on OPT accumulation. In efforts to improve OPT amount in daylily, this study was conducted to evaluate the effects of ploidy levels, genetic diversity, and growth stages on OPT concentration in leaves of daylily.

4.2. Materials and Methods

4.2.1. Plant materials and cultivation

Twenty four strains of *Hemerocallis* genus were collected from Japan, China, Taiwan, Vietnam and Bangladesh (Table 4.1, Fig. 4.1 and 4.2) and classified using the classification key for *Hemerocallis* species and cultivars described by Plodeck (2002) and Plodeck and Plodeck (2011).

All these 24 daylily strains were preserved in the experimental field of Faculty of Agriculture, University of the Ryukyus, Okinawa, Japan (26°21’ − 127°48’ E) since November 2011, adequately watered, and supplied fertilizer as nitrogen, phosphorus and potassium at 200: 200: 200 kg/ha of N: P_2O_5: K_2O, respectively.

4.2.2. Estimation of daylily ploidy

The ploidy of daylily strains was determined by Flow Cytometry method described by Saito *et al.* (2003) with some small modifications. The diploid species *H. fulva* var. *longituba*, was served as an internal standard. Basal tissues of young and health leaves of all studied daylily strains were sampled in this study.

Sample preparation was carried out following to Saito *et al.* (2003) with some small modifications. Basal tissues of leaf (ca. 5mm x 5mm) were chopped by razor blade in a petri dish containing 0.4 mL of nuclei extraction buffer (High Resolution DNA kit, CyStain UV Precise P, Partec, Germany). After incubating for 5 minutes at room temperature, extracted sample was filtered into a new tube through a 30µm nylon mesh. 1.6 mL of staining buffer (four times volume of used nuclei extraction buffer) was added to the nuclear suspension and incubated for 7 minutes at room temperature to stain. Then the relative nuclear DNA content in solution of the nuclear suspension was measured by using Cytoflow ploidy analyser Partec PA. The relative fluorescence intensity (RFI) of nuclei isolated from *H. fulva* var. *longituba* was adjusted to be at around channel 100 by instrument gain. This calibration was performed every five samples in order to minimize variation. RFI values and coefficient of variance (CV%) were recorded. The ploidy of studied daylily strains was estimated from the ratio of RFI value of sample to that of internal standard as follows:

$$Ploidy\ level = \frac{RFI\ of\ sample}{RFI\ of\ internal\ standard} \times L$$

where, L is ploidy level of plant that is used as internal standard.

4.2.3. Determination of genetic diversity of collected daylily strains based on morphological and physiological characteristics

Fourteen morphological and physiological characteristics (presented in Table 4.2) of all 24 daylily strains were recorded to evaluate the genetic diversity of collected daylily strains.

Plant height was measured from the soil surface to the arch of the uppermost leaf being at least 50% emerged from the top of plant. Plant height of 15 plants for each strain was recorded.

The measuring was carried out at the time when plant start flowering stage, because in this time, the vegetative growth of plant is the maximum.

Leaf length and leaf width were referred to the length and width of the widest leaves. Leaf length was recorded as the length of blade. And leaf width was measured at the widest point of blade. Each plant, 3 – 4 measurements was obtained, and each daylily strain, 10 plants were used.

The measurements of flowers were gathered from 10 flowers. The measured characteristics included:

- Pistil length was measured from base of ovary to top of stigmatic
- Stamen length was recorded by the length from base of filament to top of anther
- Sepal and petal length was measured from base to top of sepal and petal, excluding perianth.
- Sepal and petal width was the distance of the widest point of sepal and petal

Based on recorded data on some morphological and physiological characteristics, dendogram of 24 daylily strains was constructed using UPGMA (Unweighted Pair Group Method with Arithmetic) method in NTSYS program version 2.1.

4.2.4. Evaluation of variation of OPT concentration in daylily influenced by ploidy level and genetic diversity

Twenty four daylily strains were sampled to determine OPT concentration every month within January to December 2013. The sampling for experiments of evaluation of relationship between ploidy levels and genetic diversity and variation of OPT concentration in leaves of daylily was performed within 12 months, from January to December 2013. Monthly, each daylily

strain was harvested three samples, consisting of whole leaf of 2 – 3 plants to determine OPT concentration. The OPT concentration in the average of 12 months (three samples for each month) was shown to evaluate effects of ploidy levels on change in this compound content in leaves of daylily. And the OPT concentration in the average of strains in each group of genetic similarity was used to investigate influences of genetic diversity on OPT accumulation of daylily.

4.2.5. Evaluation of variation of OPT concentration in daylily influenced by growth stages

From 24 collected daylily strains, five 3x strains and five 2x strains were selected to evaluate the fluctuation of the OPT amount based on different growth stages, consisting of vegetative growth, flowering, and post-flowering stages. Each growth stage was observed at the same time of year among ten selected strains. During each growth stage, the sampling was conducted three times with three samples obtained each times, including total leaves of 2 − 3 plants. Namely, the vegetative growth stage was defined as the time from emerging to before budding (from roughly late December to early April), and plants were sampled at the periods of plantlet (January 2013), the most vigorous vegetative growth (February 2013), and before budding (March 2013). The flowering stage was defined as the time from budding to finishing of the flowering season (from roughly late April to late August), and plants were sampled at budding (April 2013), concentrative flowering (June 2013) and the end of the flowering season (July 2013). The post-flowering period was defined as the time from the end of the flowering season to before dormancy (from about September to early December), and the plants were sampled as soon as finishing the flowering season (August 2013), about 1 month after the flowering stage was over (September 2013), and approx. 1 month before the start of the

dormancy period (November 2013). The dormancy starts from the middle of December and lasts about 2 weeks.

4.2.6. Determination of OPT concentration

OPT concentration of daylily samples for experiments in this chapter was carried out as procedure described in chapter 2, section 2.2.7.

4.2.7. Statistical analysis

Recorded data were statistical analysis as descriptions in chapter 2, section 2.2.9.

4.3. Results and Discussion

4.3.1. Variation of OPT concentration in daylily influenced by ploidy levels

Classification of collected daylily strains and determination of ploidy level

Based on the classification key for *Hemerocallis* species and cultivars described by Plodeck (2002) and Plodeck and Plodeck (2011), 20 of 24 collected strains belonged to 4 different daylily species (Table 4.1, No. 1 – 20) and all the rest were daylily cultivars (No. 21 – 24). Of them, 9 strains of *Hemerocallis fulva* species could not be identified by their varieties, therefore they were numbered from 1 – 9.

Flow CytoMetry analysis of 24 strains of daylily revealed that 14 strains were triploid and 10 remaining strains were diploid (Table 4.1). The typical histogram of RFI of nuclei isolated from *Hemerocallis* genotypes is shown in Fig. 4.3. When the peak of RFI for the *H. fulva* var. *longituba*, being a diploid strain and used as an internal control in this study, was set at around channel 100, the peaks of that for 9 different strains were concentrated at around channel 100, and 14 other strains concentrated at around channel 150. An index was defined as the ratio of RFI of each tested strain to that of the internal control. When the index of the internal control

was set at 1.000, those of 14 different strains ranged from 1.441 to 1.593 and they were identified to be triploid. The index of 9 remaining strains ranged from 0.966 to 1.028 and they were estimated to be diploid (Table 4.1). All histograms obtained from analyzed *Hemerocallis* strains revealed a single peak with CV% value range of less than 4.0. This result was in accordance with some study results about ploidy of daylily reported. Ploidy level of *Hemerocallis* spp. may occur as diploid (2n = 2x = 22 chromosomes), triploid (2n = 3x = 33 chromosomes), and tetraploid (2n = 4x = 44 chromosomes) (Saito *et al.*, 2003; Zhang *et al.*, 2013). But tetraploid genotype has not been found in nature (Xiaobai, 1996). Also, no tetraploid was found in this study.

OPT concentration in diploid and triploid daylily strains

Results of quantitative estimation of OPT in fresh leaf of fourteen triploids and ten diploids of daylily showed that all strains accumulated OPT in ranging from 0.87 – 2.13 mg/g. Even, among 16 strains of *Hemerocallis fulva* species, two diploid strains, *H. fulva* var. *longituba* and *H. fulva* var. *littorea* did not significantly vary from almost triploids about production of OPT except for *H. fulva* var. *kwanso*, *H. fulva* var. *fulva*, *H. fulva* 'Flore Plenno', *H. fulva* 1, *H. fulva* 6 and *H. fulva* 7 (Fig. 4.4). The average OPT concentration of diploid genotypes, 1.40 mg/g, was not statistically different from that of triploid genotypes, 1.38 mg/g (Fig. 4.5). Also, based on results obtained from experiment of variation of OPT concentration influenced by growth stage of daylily, it is observed that there were no statistically difference of OPT amount between 2x and 3x daylilies (Table 4.3).

In general, polyploidy plants are considered superior to diploids in some aspects such as genetic adaptability, tolerance to environmental stresses (Estilai and Shannon, 1993), biomass

and content of effective compounds (Gao *et al.*, 1996, Griesbach and Kamo, 1996). There are several reports showing a higher amount of secondary metabolites content in polyploidy than diploid in medicinal plants. For example, Evans (1989) showed that volatile oil content in the triploid and tetraploid *Acorus calamus* was up to 50% and 300% greater than that in the diploid progenitors, respectively. In addition, morphine concentration in triploid and tetraploid types of *Papaver somniverum* was twice as those in diploids (Evans, 1996). Janaki-Ammal and Gupta (1966) demonstrated a positive proportion of essential oil concentration in leaf to polyploidy genotypes, naturally occurring 2x, 4x, and 6x of lemon grass *(Cymbopogon flexuosus)*. But there are some reports indicating the decrease of content of some secondary metabolites in polyploidy genotypes. For example, content of diosgenin in 3x and 4x *Costus speciosus* was lower than that in 2x genotype (Janaki-Ammal and Prasad, 1984). Similarly, Cellarova *et al.* (1997) showed a relationship between ploidy level and hypericin content in *Hypericum perforatum* species in which the highest hypericin content was observed in diploids and the lowest in tetraploids, and it was assumed that difference in density of multi-cellular glands, where hypericin is synthesized and accumulated, resulted in difference in hypericin content in 2x and 4x genotypes of this plant. These results showed that ploidy levels affects significantly on quantitative changes of plant chemicals. This effect differed between plant species, secondary metabolite type, growth form, presence of synergy and other factors (Koricheva, 2002, Nykanen and Koricheva, 2004). However, report of Griesbach and Kamo (1996) pointed out that the haploid, diploid and tetraploid genotypes of *Petunia* 'Mitchel' did not vary about the total concentration of floral flavonols and concentration of four other minor flavonols. Using meta-analysis method of data from 96 experiments in 74 publications concerning 21 families and 36 genera of plants, Massad

et al. (2011) showed that genetic manipulations affected significantly on the concentration of C-based secondary metabolites, but did not affected on N-based secondary metabolites, and woody plant chemicals responded to genetic factors, but herbaceous plant secondary metabolites did not. In this study, the accumulation of OPT, a nitrogen compound as shown in Fig. 2.1 (Ogawa *et al.*, 2013), was not affected by ploidy levels of *Hemerocallis* spp, however, variation of the concentration of OPT was different in each ploidy level. While OPT concentration of triploid strains were quite uniform, from 1.22 to 1.60 mg/g FL, this value was widely variable in diploids, from 0.87 to 2.13 mg/g (Fig. 4.4). This issue was also expressed clearly by higher SE value of the means of diploid populations than that of triploid populations (Fig. 4.5, Table 4.3). It is assumed that the difference of variation of OPT between 2x and 3x daylily was obtained because studied diploid strains included different species and cultivars, whereas all triploid strains were *H. fulva* species. It was also observed that, the OPT concentration of two diploid strains of *H. fulva* species (*H. fulva* var. *longituba* and *H. fulva* var. *littorea*) was lower than that of the rest triploid *H. fulva* strains, even significantly lower than that of six of 14 triploid *H. fulva* strains (*H. fulva* var. *kwanso, H. fulva* var. *fulva*, *H. fulva* 'Flore Plenno', *H. fulva* 1, *H. fulva* 6 and *H. fulva* 7), this result suggests that OPT concentration in diploid genotypes could be improved by artificial polyploidization of diploid genotypes. The influence of artificially induced polyploidy on OPT concentration in daylily was not evaluated by this study and requires further elucidation.

4.3.2. Variation of OPT concentration in daylily influenced by genetic diversity

Based on recorded data in morphological and physiological characteristics (Table 4.2), 24 daylily strains were grouped using UPGMA method. The results of this analysis were presented in Fig. 4.6. At level about 7.5 of similarity coefficient, 24 studied daylily strains could be divided

into 7 main clusters, marked by number from 1–7 in Fig. 4.6. At level of 6.45 of similarity coefficient the group No. 4 also was divided into 2 sub-clusters (4.1 and 4.2) to support for evaluation of variation of OPT concentration in leaves in different daylily clusters. The OPT concentration of these eight daylily clusters was showed in Fig. 4.7. A significant difference in OPT amount was observed between cluster 1, consisting only one daylily strain (*H.* 'Stella de Oro') and the rest groups. Although the OPT accumulation of seven other groups were not significantly different, recorded data presented in Fig. 4.4 showed that the different strains in the same species such as strains of species *H. fulva* and *H. citrina* and the different species accumulated the different OPT amount.

Relationship between genetic and phytochemical constituents and quantity have been studied in some medicinal plants, such as thyme (*Thymus vulgaris*) (Vernet *et al.*, 1986, Echeverrigaray *et al.*, 2001), *Ocimum gratissimum* (Vieira *et al*., 2001), *Tanacetum vulgare* (Keskitalo *et al*., 2001). And almost all showed the high correlation between genetic and chemical didtance matries.

4.3.3. Variation of OPT concentration in daylily influenced by growth stages

The concentration of OPT in daylily leaves varied clearly with the three growth stages (Table 4.3). All of the studied strains showed the lowest OPT concentration during the post-flowering period, ranging from 0.42 to 1.68 mg/g. Higher OPT concentrations were accumulated during the vegetative and flowering stages with the range 1.29 − 2.79 mg/g and 1.27 − 2.15 mg/g, respectively. The OPT production of the daylilies at two these stages did not vary, except for the *H. fulva* var. *kwanso* and *H. aurantica* 'Major' strains.

Variations of secondary metabolites in plants as influenced by phenological stages have been demonstrated in many researches. In the species *Hypericum perforatum*, the highest content of hypericin was accumulated at the full flowering stage, then immediately declined (Brantner *et al*., 1994). Also, Cirak *et al.* (2008) and Radušienė *et al*. (2012) reported that in *Hypericum perforatum*, the production of some phenolic compounds including apigenin-7-O-glucoside, quercitrin, quercetin, chlorogenic acid, rutin, hyperoside and total phenolics varied greatly during phenological phases, and the highest amount of these compounds was produced at the flowering period. At the vegetative growth stage, the contents of these compounds were slightly lower but not significantly different from those at the flowering stage. By the study of changes in content of main secondary metabolites in two species of Turkish *Hypericum* during plant development, Cirak *et al*. (2013) reported that species of *Hypericum* should be harvested during flower ontogenesis for medicinal purposes, because the content of many bioactive compounds was the highest in this phase. They also showed that at the full flowering stage, the highest contents of hypericin, pseudohypericin, and quercitrin were found in the species *Hypericum avicullariifolium* whereas in the species *Hypericum orientale* at fresh fruiting, the maximum value of quercitrin was detected and no presence of hypericin and pseudohypericin was found. From results of study of the variation of content and composition of essential oil and phenolic compounds in sweet marjoram (*Origanum majorana* L.) at four growth periods, Sellamia *et al.* (2009) pointed out that at the late vegetative growth stage, the phenolic concentration was the highest whereas the essential oil yield was the lowest. Those authors proposed that during the late vegetative stage, *O. majorana* plants are protected from unfavorable conditions mainly by phenolics. Moreover, at this stage, phenolics are accumulated in plants as a part of the preparation for the process of

lignification that occurrs immediately after this stage is completed. The highest yield of essential oil during the flowering stage was thought to be related to ecological function such as pollinator attraction and antifungal defense intensification (Langenheim, 1994).

Clearly, the results of the above-described studies indicate that particular plant species at different growth stages may contain significantly different contents of specific secondary metabolites. In the case of *Hemerocallis* spp., the vegetative and flowering stages may be favoured to harvest for OPT purposes. At post-flowering, plants grow slowly as they enter dormancy, and this is thought to be linked to the decrease in OPT at this growth stage.

Table 4.1 Ploidy level of the 24 collected *Hemerocallis* strains

No.	Species/cultivar name	Locality of collection	RFI	RFI ratio	Ploidy level
1	*Hemerocallis fulva* var. *sempervirens*	Okinawa, Japan	159.49	1.593	3×
2	*Hemerocallis fulva* var. *kwanso*	Kyushu, Japan	145.84	1.457	3×
3	*Hemerocallis fulva* var. *fulva*	Okinawa, Japan	146.85	1.467	3×
4	*Hemerocallis fulva* 'Hankow'	Taitung, Taiwan	151.25	1.511	3×
5	*Hemerocallis fulva* 'Flore Pleno'	Dhaka, Bangladesh	150.73	1.506	3×
6	*Hemerocallis fulva* 1	Hualien, Taiwan	150.22	1.501	3×
7	*Hemerocallis fulva* 2	Hualien, Taiwan	154.31	1.541	3×
8	*Hemerocallis fulva* 3	Hualien, Taiwan	157.84	1.577	3×
9	*Hemerocallis fulva* 4	Okinawa, Japan	155.23	1.551	3×
10	*Hemerocallis fulva* 5	Kagoshima, Japan	147.13	1.470	3×
11	*Hemerocallis fulva* 6	Taitung, Taiwan	145.68	1.455	3×
12	*Hemerocallis fulva* 7	Hokkaido, Japan	157.12	1.569	3×
13	*Hemerocallis fulva* 8	Thai Nguyen, Viet Nam	148.79	1.486	3×
14	*Hemerocallis fulva* 9	Dhaka, Bangladesh	144.23	1.441	3×
15	*Hemerocallis fulva* var. *littorea*	Ibaraki, Japan	98.25	0.981	2×
16	*Hemerocallis fulva* var. *longituba**	Okinawa, Japan	100.11	1.000	2×
17	*Hemerocallis aurantiaca* 'Major'	Taitung, Taiwan	97.82	0.977	2×
18	*Hemerocallis citrina* var. *vespertina*	Hunan, China	100.96	1.008	2×
19	*Hemerocallis citrina*	Hubei, China	100.71	1.006	2×
20	*Hemerocallis altissima*	Osaka, Japan	100.26	1.001	2×
21	*Hemerocallis* 'Stella de Oro'	Beijing, China	100.93	1.008	2×
22	*Hemerocallis* 'Red Bird'	Okinawa, Japan	102.95	1.028	2×
23	*Hemerocallis* 'Black Eyed Stella'	Okinawa, Japan	101.28	1.012	2×
24	*Hemerocallis* 'Margaret Perry'	Okinawa, Japan	96.72	0.966	2×

Notes: The RFI data are mean values of triplicate experiments.

* This strain was used as an internal standard.

Table 4.2 Morphological and physiological characteristics of daylily used for genetic diversity analysis

Hemerocallis spp. name	Plant height (cm)	Leaf length (cm)	Leaf width (cm)	Pistil length (mm)	Statmen length (mm)	Sepal length (mm)	Sepal width (mm)	Petal length (mm)	Petal width (mm)	Flower type	Flower color	Flowering time	Blooming season	Dormancy
H. fulva var. *sempervirens*	32.2	46.4	1.36	124.60	82.25	86.67	20.00	88.67	20.50	Single	Orange	Diurnal	Aug -Nov	Evergreen
H. fulva var. *kwanso*	38.0	58.2	2.02	123.00	71.42	88.00	21.67	88.50	30.00	Double	Orange	Diurnal	May -Aug	Dormant
H. fulva var. *fulva*	32.2	69.4	2.31	115.40	74.23	81.67	18.17	80.50	29.33	Single	Orange	Diurnal	April -Jul	Dormant
H. fulva 'Hankow'	41.8	71.4	2.60	124.40	72.75	88.33	21.83	90.50	34.50	Single	Orange	Diurnal	April -Aug	Dormant
H. fulva 'Flore Pleno'	36.2	56.2	1.98	117.40	71.92	88.00	21.67	89.00	30.50	Double	Orange	Diurnal	May -Aug	Dormant
H. fulva 1	27.8	40.4	1.42	124.80	81.17	87.17	20.00	89.00	20.17	Single	Orange	Diurnal	Aug -Nov	Evergreen
H. fulva 2	26.8	37.6	1.36	124.60	82.33	88.17	20.50	88.67	20.00	Single	Orange	Diurnal	Aug -Nov	Evergreen
H. fulva 3	27.8	36.2	1.36	125.00	82.67	87.50	20.00	88.50	19.50	Single	Orange	Diurnal	Aug -Nov	Evergreen
H. fulva 4	28.2	39.4	1.46	127.00	83.00	87.17	19.67	88.17	20.17	Single	Orange	Diurnal	Aug -Nov	Evergreen

Table 4.2 Morphological and physiological characteristics of daylily used for genetic diversity analysis (Continued)

Hemerocallis spp. name	Plant height (cm)	Leave length (cm)	Leave width (cm)	Pistil length (mm)	Statmen length (mm)	Sepal length (mm)	Sepal width (mm)	Petal length (mm)	Petal width (mm)	Flower type	Flower color	Flowering time	Bloom season	Dormancy
H. fulva 5	26.0	41.0	1.38	125.60	81.58	87.33	20.00	88.17	20.33	Single	Orange	Diurnal	Aug -Nov	Evergreen
H. fulva 6	21.0	28.2	1.08	125.80	82.00	87.50	20.33	88.33	20.33	Single	Orange	Diurnal	Aug -Nov	Evergreen
H. fulva 7	24.8	45.6	1.58	118.20	76.33	91.33	21.17	95.33	24.00	Single	Orange	Diurnal	May - Jul	Dormant
H. fulva 8	42.0	68.8	2.28	117.00	71.25	99.00	18.00	99.00	27.33	Single	Orange	Diurnal	April - Jun	Dormant
H. fulva 9	29.0	46.6	1.92	137.20	73.83	99.50	17.67	101.50	27.17	Single	Orange	Diurnal	May -Aug	Dormant
H. fulva var. *littorea*	28.3	50.6	1.97											Dormant
H. fulva var. *longituba*	22.6	51.0	1.70	110.00	72.55	92.50	17.50	97.00	30.00	Single	Orange	Diurnal	May - Oct	Dormant
H. aurantiaca 'Major'	29.6	66.4	2.34	91.20	58.92	105.00	22.50	107.50	39.00	Single	Yellow	Nocturnal	Mar - Aug	Dormant
H. citrina var. *vespertina*	40.8	56.5	1.57	152.10	69.75	98.67	11.92	116.83	20.83	Single	Pale yellow	Nocturnal	May -Aug	Dormant
H. citrina	43.0	60.2	1.70	156.40	84.50	112.33	12.67	118.50	21.67	Single	Pale yellow	Nocturnal	May -Aug	Dormant

Table 4.2 Morphological and physiological characteristics of daylily used for genetic diversity analysis (Continued)

Hemerocallis spp. name	Plant height (cm)	Leave length (cm)	Leave width (cm)	Pistil length (mm)	Statmen length (mm)	Sepal length (mm)	Sepal width (mm)	Petal length (mm)	Petal width (mm)	Flower type	Flower color	Flowering time	Bloom season	Dormancy
H. altissima	14.6	55.0	1.70	104.60	57.67	78.50	10.50	82.67	18.67	Single	Pale yellow	Nocturnal	May - Oct	Dormant
H. 'Stella de Oro'	23.2	29.8	1.14	78.20	42.17	59.67	20.50	58.17	32.00	Single	Yellow	Diurnal	Mar - Jul	Dormant
H. 'Red Bird'	33.2	30.6	1.22	114.60	67.42	105.00	25.00	103.50	38.00	Single	Reddish	Nocturnal	May - Jul	Dormant
H. 'Black Eyed Stella'	36.6	50.4	1.36	111.60	66.42	79.00	14.33	84.50	20.83	Single	Yellow with eyed black	Diurnal	April - Jun	Evergreen
H. 'Margaret Perry'	28.8	72.8	2.36	116.40	68.75	106.67	28.50	106.50	37.50	Single	Dark red	Diurnal	Mar - Aug	Evergreen

Notes: Dormant: The leaves of these daylily strains die completely when winter approaches. They stop growing and form resting buds at the crown, and the foliage dies down naturally and gradually. After time of over winter, the resting buds have a distinctive spear-like appearance as they emerge. Evergreen: These daylilies retain their leaves throughout the year. They continually produce new leaves unless cold weather prevents growth.

Table 4.3 The OPT concentrations in *Hemerocallis* spp. leaves during growth stages

Hemerocallis strains	Ploidy level	OPT concentration (mg/g FL)		
		Vegetative growth stage	Flowering stage	Post-flowering stage
Hemerocallis fulva 7	3×	1.69 ± 0.02 ^{bcA}	1.89 ± 0.08 ^{abA}	1.37 ± 0.14 ^{abB}
Hemerocallis fulva 8	3×	1.39 ± 0.27 ^{cdA}	1.88 ± 0.21 ^{abA}	0.42 ± 0.06 ^{eB}
Hemerocallis fulva 'Flore Pleno'	3×	1.73 ± 0.04 ^{bcA}	1.86 ± 0.16 ^{abA}	1.14 ± 0.06 ^{bcB}
Hemerocallis fulva var. *kwanso*	3×	1.40 ± 0.01 ^{cdB}	1.78 ± 0.15 ^{abA}	1.15 ± 0.08 ^{bcB}
Hemerocallis fulva 'Hankow'	3×	1.44 ± 0.18 ^{cdA}	1.68 ± 0.16 ^{bcA}	0.84 ± 0.07 ^{cdB}
Mean		**1.53 ± 0.07 ^{aA}**	**1.82 ± 0.04 ^{aA}**	**0.98 ± 0.16 ^{aB}**
Hemerocallis aurantica 'Major'	2×	1.53 ± 0.12 ^{bcB}	1.85 ± 0.11 ^{abA}	0.80 ± 0.08 ^{dC}
Hemerocallis citrina	2×	1.29 ± 0.03 ^{dA}	1.27 ± 0.07 ^{cA}	1.15 ± 0.10 ^{bcA}
Hemerocallis 'Stella de Oro'	2×	2.79 ± 0.28 ^{aA}	2.01 ± 0.31 ^{abA}	1.68 ± 0.14 ^{aB}
Hemerocallis citrina var. *vespertina*	2×	1.98 ± 0.24 ^{bA}	2.15 ± 0.02 ^{aA}	1.28 ± 0.02 ^{bB}
Hemerocallis 'Black Eyed Stella'	2×	1.58 ± 0.12 ^{bcA}	1.51 ± 0.04 ^{bcA}	0.91 ± 0.01 ^{cdB}
Mean		**1.84 ± 0.26 ^{aA}**	**1.76 ± 0.16 ^{abA}**	**1.16 ± 0.15 ^{aB}**

Notes: Data are means ±SE (standard errors) (n = 9; 3 sampling times with 3 samples obtained each times). In each column, strains with data marked by the same superscript lowercase letters are not significantly different by LSD (P<0.05). In each row, growth stages with data marked by the same superscript uppercase letters are not significantly different by LSD ($P<0.05$).

Fig. 4.1 Diploid strains of *Hemerocallis* spp..

Fig. 4.2 Triploid strains of *Hemerocallis* spp..

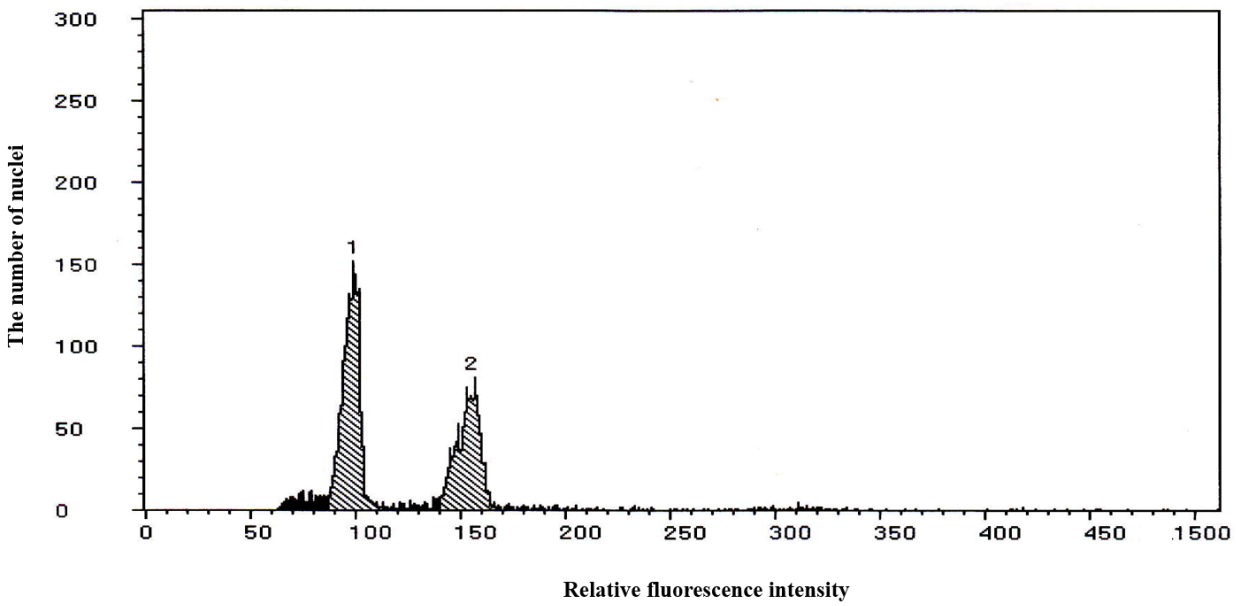

Fig. 4.3 Histogram from the flow cytometry analysis of the nuclear DNA content of *H. fulva* var. *longituba* (diploid) (peak 1) and *H. fulva* var. *sempervirens* (triploid) (peak 2).

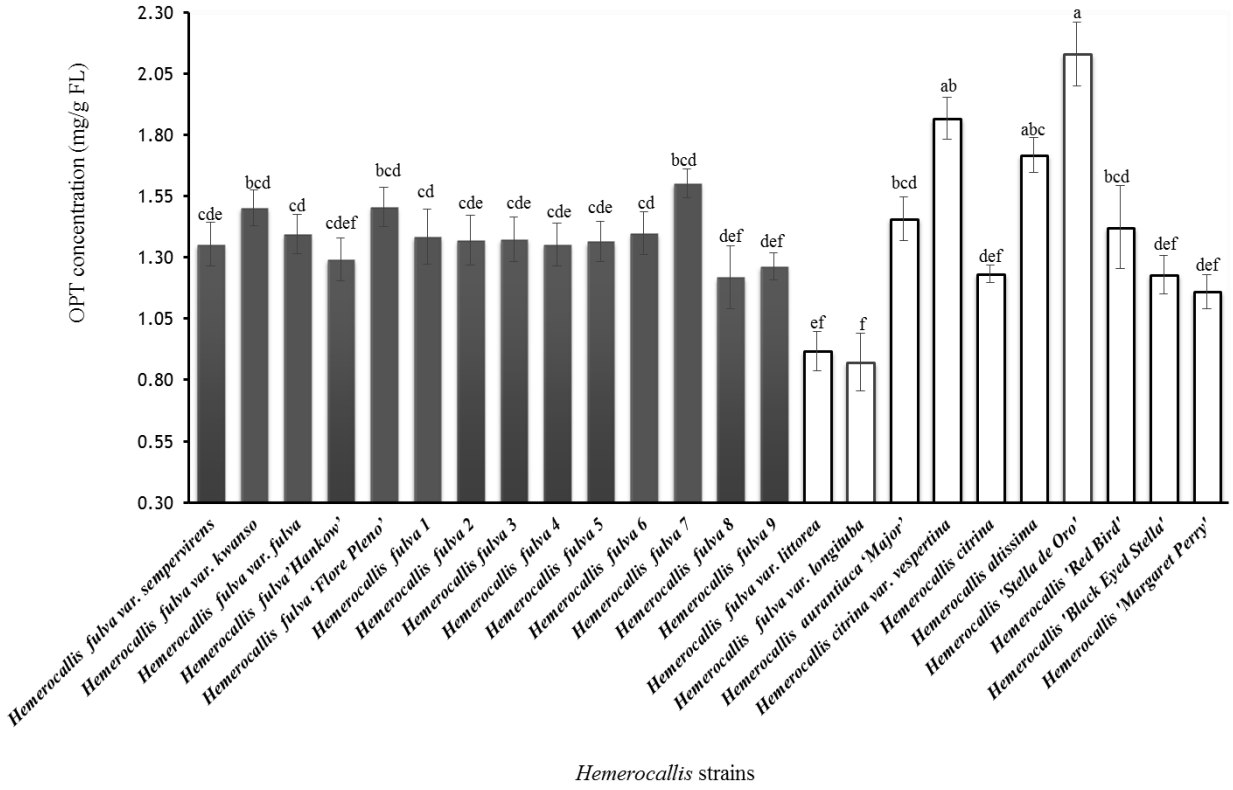

Fig. 4.4 The OPT concentrations in triploid (filled columns) and diploid (non-filled columns) *Hemerocallis* strains.

Notes**:** The columns indicate the OPT concentrations of *Hemerocallis* strains as the average of 12 months. The vertical bar on each column represents the standard error (SE) value of the mean (n=36; 12 months with 3 samples each month). Columns with the same lowercase letters are not significantly different by LSD ($P<0.05$).

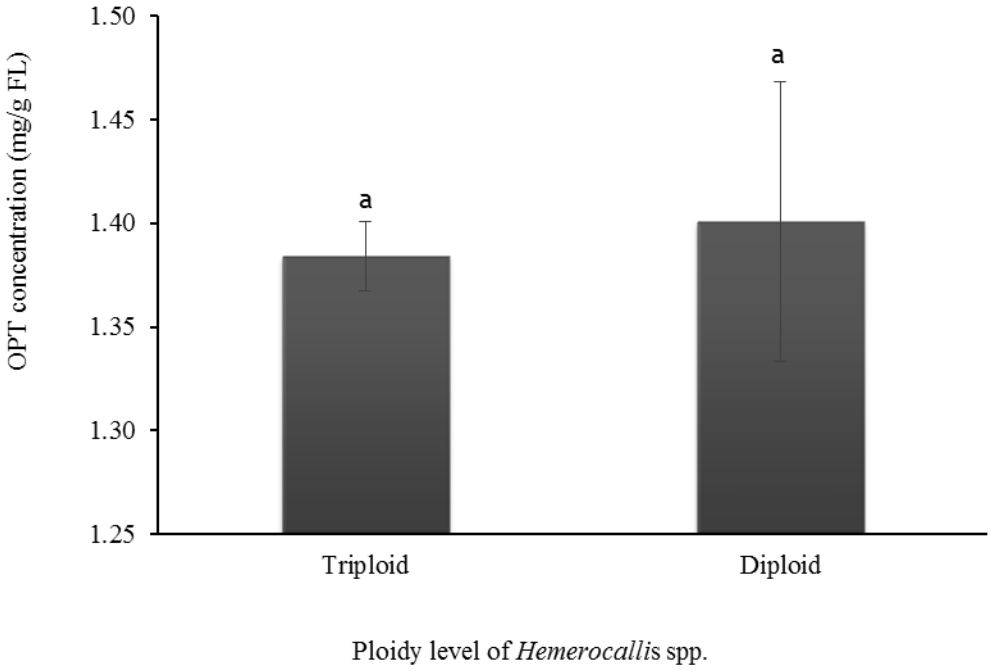

Fig. 4.5 OPT concentration in diploid (2x) and triploid (3x) daylily strains.

Notes: The columns indicate the average OPT concentration of 14 triploid daylily strains and of 10 diploid daylily strains. The vertical bar on each column represents the standard error (SE) value of the mean (n =14 with 3x strains and n = 10 with 2x strains). The columns with the same lowercase letters are not significantly different by LSD ($P<0.05$).

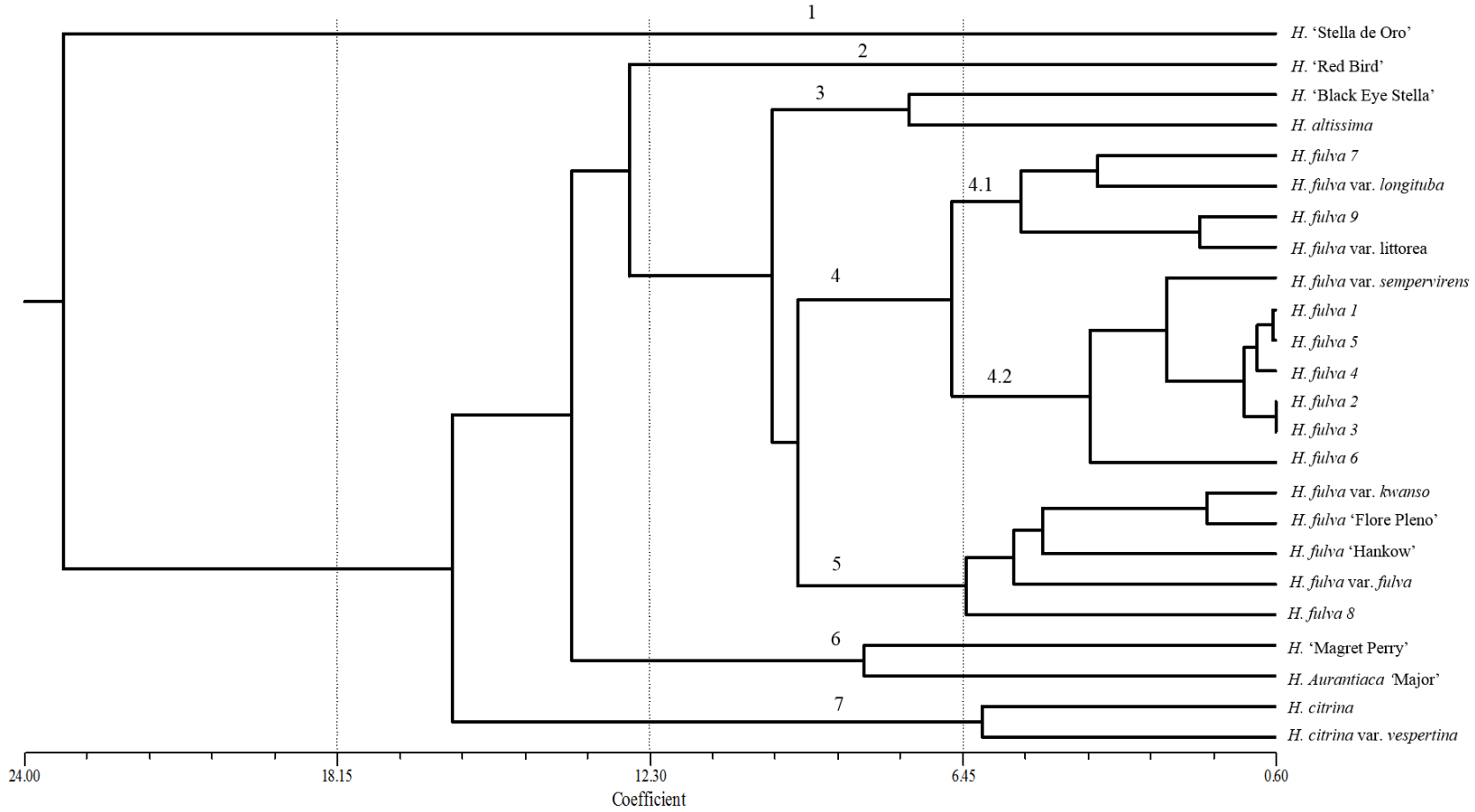

Fig. 4.6 Dendogram from UPGMA cluster analysis based on some morphological and physiological characteristics of 24 collected daylily strains.

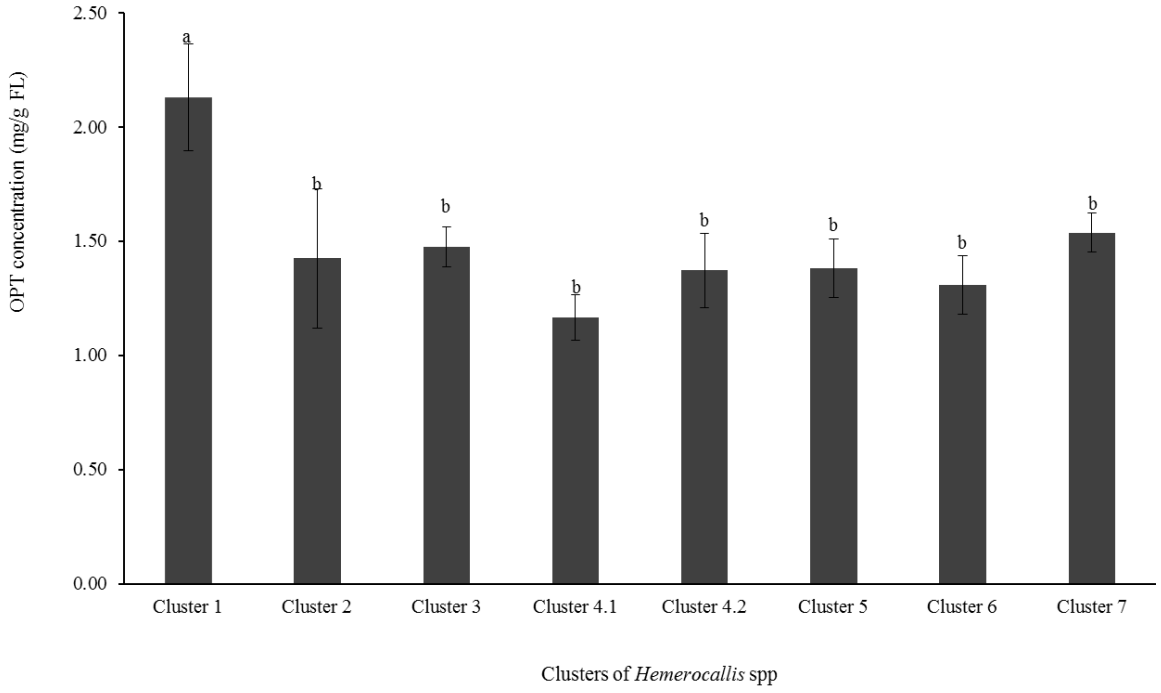

Fig. 4.7 OPT concentration in differently genetic similarity clusters of daylily.

CHAPTER 5

Preliminary investigation of secondary metabolites and antioxidant capacity of methanol extracts from daylily (*Hemerocallis* spp.)

5.1. Introduction

Plant secondary metabolites are a large and diverse array of organic compounds produced by plants. These compounds are also known to play a crucial role in the adaptation of plants to their environment and show an important source of active pharmaceuticals. Pharmaceutically significant secondary metabolites or called phytopharmaceuticals include alkaloids, glycosides, phenolic, flavonoid, tannin, resin, volatile oils etc. Higher plants, used for traditional medicine to treat many human diseases are the rich source of phytopharmaceuticals used in pharmaceutical industry. According to the WHO, about 88% of world population relies mainly on traditional medicine for their primary health care (WHO, 1999). In modern times, plant secondary metabolites have been isolated for drug discovery and development. Currently, one-fourth of all prescribed pharmaceutical in industrialized countries contain compounds those are derived from plants via semi-synthesis directly or indirectly (Namdeo, 2007). Prescription drugs containing phytochemicals were valued at more than 30 billion US dollars in 2002 in the USA alone (Raskin *et al.*, 2002). Although phytopharmaceuticals possess many beneficial bioactivities and have been demonstrated highly pharmacological values, most of them still only isolated from medicinal plants. The synthesis of these compounds is either extremely difficult or economically infeasible because of their highly complex chemical structures and the specific stereo-chemical

requirements. Thus the touting phytochemical profiles and their bioactivities in medicinal plants in order to supply to known phytopharmaceutical list is necessary.

Oxidation is thought to induce cellular damages linking to contamination and progression of several human diseases such as cancer, arteriosclerosis, cardiovascular, inflammatory, neural degenerative disorders as well as aging related disorders (Perry *et al.*, 2000, Singh and Singh, 2008). Free radicals generation is related directly with oxidation in foods and biological systems. And antioxidants are known to have a significant influence on the status of human health and disease prevention (Bland, 1995). Dasgupta *et al.* (2007) reported that the damage effects of oxidation types such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals, and peroxynitrite on cells are abrogated by plant antioxidant compounds. Thus free radical scavenging ability is used to determine the antioxidant capacity of antioxidant compounds. Medicinal plants, having antioxidant activity or free radical scavenging effect, have been used as the sources for developing new phytochemicals for the treatment and prevention of these diseases (Cichewicz and Nair, 2002, Zhang *et al.*, 2004, Wang *et al.*, 2005, Tiwari and Tripathi, 2007).

DPPH (1,1-diphenyl, 2-picryl hydrazyl) is a well-known radical, composed by stable free radical molecular, and an efficient scavenger for other radicals (https://en.wikipedia.org/wiki/DPPH). Therefore, DPPH free radical scavenging assay offers the first approach for evaluating the antioxidant potential of a compound, an extract or other biological sources (Kedare and Singh, 2011).

Daylily (*Hemerocallis* spp.), a perennial medicinal plant, have been used in traditional medicine to treat many human diseases and is also reported to exhibit striking activities against the human pathogenic trematode *Schistosoma mansoni* (Cichewicz *et al*., 2002) and several

human cancer cell lines (Cichewicz *et al.*, 2004). Phytochemical extracts of daylily contain a great number of secondary metabolites which have been demonstrated effects for treatment several diseases, including anthocyanins, flavonoids, carotenoids, amino acid amides and potential agents for the inhibition of lipid oxidation, etc. (Thu *et al.*, 2004, Zhang *et al.*, 2004, Mao *et al.*, 2006, Ogawa and Konishi, 2009, Urade *et al*., 2010, Ogawa *et al*., 2013). This study was carried out to investigate more about the phytochemical constituents and antioxidant capacity in extracts of some daylily strains in order to know the medicinal benefits and pharmaceutical potential of daylily.

5.2. Materials and Methods

5.2.1. Plant materials

Three strains of daylily were chosen for this study, they are *H. sempervirens, H. citrina*, and *H.* 'Stella de Oro' (Fig. 5.1.). *H. sempervirens* is the triploid species, used commonly in Okinawa to treat insomnia. The flower color is orange, flower opening time of this species is diurnal, and root structure of *H. sempervirens* is rhizomatous; *H. citrina* is the diploid dalily species. Its flower color is pale yellow, the opening time is nocturnal, and root structure is fibrous; and *H.* 'Stella de Oro' is a small-growing cultivar, which would be ideal for use in scientific growth rooms, or glasshouses where space is limited. *H.* 'Stella de Oro' is characterized as a diploid cultivar, its flower color is yellow, flowering time is diurnal, and root structure is fibrous. These three daylilies were collected from Japan and China, and preserved in experimental area of Faculty of Agriculture, University of the Ryukyus, Okinawa from 2011.

5.2.2. Collection of samples and extraction

Fresh leaves, roots, and flowers of three selected *Hemerocallis* accessions were sampled randomly. Next, these samples were washed under running tap water to remove the surface

pollutants and air dried at room temperature. Then they were ground and extracted using aqueous methanol (70% v/v) for 72 h at room temperature. The extract solutions were filtered through Whatman filter paper No. 6. The filtrate was evaporated to remove completely solvents using system of rotary vacuum evaporator. The dry residues obtained after evaporating were weighed to determine the extraction yield, and then they were stored at 0 – 4ºC until the time of analysis. The yield of extraction was presented in Table 5.1

5.2.3. Detection of secondary metabolites in daylily

Although the secondary metabolites extracted from plants are very diverse with thousands identified chemical structures, possessing interesting pharmaceutical activities, they can be divided into three main groups based on their biosynthetic origins: alkaloids or nitrogen containing compounds, terpenoids, and phenolics (Verpoorte, 1998, Savithramma *et al.*, 2011, Justin *et al.*, 2014).

In this study, methanol extracts prepared from leaves, roots, and flowers of daylilies were preliminarily screened for constituents of secondary metabolites, consisting of alkaloids of alkaloids group; terpenoids, steroids, glycosides, cardiac glycosides, anthraquinone, and saponin of terpenoids group; phenolics, tannins, flavonoids, anthocyanin, leucoanthocyanin, and coumarins of phenolics group by the following standard methods with minor modifications.

Detection of alkaloids

Wagner's test: 0.5 mL of concentrated sunfuric acid was added to 1 mL of aqueous extract. Then 2 – 3 drops of Wagner reagent (prepared by dissolving 2 g of iodine and 6 g of potassium iodine in 100 mL H_2O) were added to above mixture. A brown precipitate indicates the presence of alkaloids.

Detection of terpenoids

Salkowski's test: 0.5 g of extract was dissolved in 2 mL of chloroform and filtered. The filtrate wad added carefully by equal volume of concentrated sulphuric acid to form a layer. The formation of reddish brown ring in interface shows the presence of terpenoids (Ayoola *et al.*, 2008).

Detection of steriods

Salkowski's test: 2 mL of extract solution was added with 5 mL of chloroform. The equal volume of concentrated sulphuric acid was added to above mixture along the wall of the test tube. The test tubes were shaken for few minutes. The red coloration in the upper layer and yellow coloration with green fluorescence in lower layer shows the presence of the steroids (Gibbs, 1974, Geetha and Geetha, 2014).

Detection of glycosides

About 1 g of extract dryness was dissolved with 2 mL of water. Then 10% aqueous NaOH was added. The formation of yellow color indicates the presence of glycosides (Geetha and Geetha, 2014).

Keller-Killiani's test for cardiac glycosides: One (1) mL of extract solution was mixed with 5 mL of 70% aqueous ethanol for 2 min and filtered. The filtrate was diluted with 10 mL of Milli Q water and added by 0.5 mL of 5% lead acetate. This solution was filtered and the obtained filtrate was shaken with equal volume of chloroform. The chloroform layer was obtained and evaporated to dryness on water bath. The residue was cooled and dissolved in 3 mL of glacial acetic acid containing 2 drops of 5% $FeCl_3$ solution. Then this solution was transferred into a test tube and about 2 mL of concentrated sulphuric acid were added along the wall of test tube. On standing, a reddish brown layer was presence between two liquid phases, the upper phase turns slowly pale green and darkening with standing indicates the presence of cardiac glycosides

(Harborne, 1984, Ben *et al*., 2013).

Borntrager's test for anthraquinone glycosides: 1 mL of extract solution was added by few drops of 10% sulphuric acid. This mixture was boiled and filtered. About 2 mL of chloroform was added to the filtrate and shaken well for 5 min. The chloroform layer was separated and added slowly by equal volume of 10% ammonia solution with gentle shake. The appearance of red color of ammoniacal layer points out the presence of anthraquinone glycosides (Harborne, 1984, Sofowora, 1993).

Detection of saponins

0.5 g of extract was diluted with 10 ml distilled water. Then few drops of 5% sodium bicarbonate solution were added to the extract solution. The mixture was shaken well in a graduated cylinder for 15 min. The formation of froth layer indicates the presence of saponins (Kumar *et al.*, 2009, Tiwari *et al.*, 2011).

Detection of tannins and phenolics

About 1 g of extract residue was dissolved in 25 mL of Milli-Q water and warmed in boiled water bath for 5 min. Then it was cooled, filtered. The filtrate was adjusted with Milli-Q water to 25 mL. This extract solution was used for detection of tannin and phenols as methods described by Geetha and Geetha (2014) with the small modifications.

Lead acetate's test: 2 mL of extract solution was added with 5 – 6 drops of 1% lead acetate solution. The formation of yellow precipitate shows the presence of tannins and phenolic compounds.

Ferric chloride test for tannins: 0.5 mL of neutral 5% ferric chloride solution was added to 2 mL of extract solution. The formation of dark bluish black color indicates the presence of tannins. Neutral 5% ferric chloride was prepared by dissolving 5 g of $FeCl_3$ with 70 mL of 90% aqueous

alcohol, and then the volume was adjusted to 100 mL by 90% aqueous alcohol.

Sodium hydroxide test for phenolics: 2 mL of extract solution was added with 1mL of 20% sulphuric acid solution, followed by addition of few drops of 10% aqueous NaOH solution. The formation of blue color indicates the presence of phenolic.

Detection of flavonoids

Lead acetate test: 2 mL of extract solution was added with 1mL of 10% aqueous lead acetate solution. The formation of yellow precipitate is positive for flavonoids (Tiwari *et al.*, 2011, Geetha and Geetha, 2014)

Detection of anthocyanins

Two (2) mL of aqueous extract was added with 2 mL of 2N HCl and 2 mL of 10% ammonia. The appearance of turning from pink-red to blue-violet color indicates the presence of anthocyanins (Savithramma *et al*., 2011).

Detection of leucoanthocyanins

Five (5) ml of aqueous extract were added to 5 ml of isoamyl alcohol. The appearance of red color in upper layer indicates for presence of leucoanthocyanins (Savithramma *et al*., 2011).

Detection of coumarins

Two (2) mL of aqueous extract was added with 2 mL of 10% NaOH. The formation of yellow color is positive for coumarins (Savithramma *et al.*, 2011).

5.2.4. Determination of total phenolic content

Total phenolic content in the methanol extracts of roots, leaves, and flowers of three selected daylily strains was determinate by using the Folin-ciocalteu method as described by Singleton *et al.* (1965) with small modifications. A total of 100 mg of dryness extract was dissolved in Milli-Q water and the volume was adjusted to 100 mL using Milli-Q water. Gallic

acid was used as standard compound to make a calibration curve. H_2O was used to prepare the reagent blank. Briefly, 1 mL of aqueous extract (1 mg/mL), or gallic acid solution at concentration of 20, 40, 60, 80, 100, 250, 500 µg/mL, or H_2O was taken in a test tube. To this solution, 5 mL of Milli-Q water and 1 mL of Folin-ciocalteu reagent were added and the mixture was shaken well for 5 min. After that, 1 mL of 20% Na_2CO_3 solution was added and this reaction mixture was incubated at room temperature for 2 h with gentle shaking. The absorbance of reaction mixture was measured against prepared reagent blank at 765 nm of wavelength using a Shimadzu UV-visible spectrophotometer model PharmaSpec UV-1700. The total phenolic content was expressed as gallic acid equivalent (GAE) in mg/g extract. Each sample was analyzed in triplicates.

5.2.5. Determination of total flavonoid content

Total flavonoid content in the methanol extracts of roots, leaves, and flowers of the selected daylily strains was measured spectrophotometrically by aluminium chloride colorimetric assay described by Marinova *et al.*, (2005) with slight modifications. A total of 100 mg of extract dryness was dissolved and adjusted in volume to 100 mL by 70% aqueous methanol. Catechin was used to make a calibration curve. 70% aqueous methanol was used to make the reagent blank. Briefly, 1 mL of extract solution (1 mg/mL), or catechin solution with different concentrations such as 25, 50, 100, 250, 500 µg/mL, or 70% methanol was added with 4 mL of Milli-Q water. To this mixture, 0.3 mL of 5% NaNO2 was added and mixed. After 5 min, 0.3 mL of 10% $AlCl_3$ solution was added and mixture was mixed for 5 min. At 6th min, 2 mL of 1M NaOH solution was added and the volume was made up to 10 mL with Milli-Q water. This reaction mixture was mixed well for 15 min at room temperature and the absorbance was measured against prepared reagent blank at 510 nm by using a Shimadzu UV-visible

spectrophotometer model PharmaSpec UV-1700. The total flavonoid content was expressed as catechin equivalent (CE) in mg/g extract. Each analysis was conducted in triplicates.

5.2.6. Investigation of antioxidant activity of methanol daylily extracts in DPPH radical scavenging assay

The scavenging of the stable 2, 2-diphenyl-1-picryl-hydrazil (DPPH) radical is the most popular method used widely to evaluate the free radical scavenging ability of various samples, including plant extracts (Chang *et al.*, 2002). In this study, the DPPH radical scavenging assay for daylily extract was performed as described by Hatano *et al.*, (1988) with a few modifications. Butylated Hydroxy Toluene (BHT), gallic acid, and catechin were used as the standard DPPH free radical scavengers (positive controls). Methanol (99.8% type) was used to prepare negative control. One (1) mL of extract solution dissolved in methanol or positive control at different concentrations (25, 50, 100 µg/mL), was added to a test tube containing 0.5 mL of DPPH (0.5 mM in methanol), and 1 mL sodium acetate buffer (0.1 M, pH 5.5). The reaction mixture was incubated at room temperature in dark for 30 min. The absorbance was measured at 517 nm using a Shimadzu UV-visible spectrophotometer model PharmaSpec UV-1700. Each analysis was conducted in triplicates. The percentage radical scavenging capacity was determined using the following formula:

$$\text{DPPH Scavenging (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

From calibration curve with sample concentration along *X*-axis and % DPPH scavenging along *Y*-axis, and effect concentration value of samples that scavenges 50% of the DPPH radical (EC_{50} (µg/mL) was calculated. EC_{50} value is inversely proportional to antioxidant capacity of compound

4.2.7. Statistical analysis

Data of this experiment were analyzed statistically by methods described in Chapter 2 section 2.2.9.

5.3. Results and Discussion

5.3.1. Secondary metabolite constituents in daylily extracts

The results of qualitative analysis of phytochemicals in root, leaf, and flower extracts of three selected daylily strains (*H. sempervirens, H. citrina*, and *H.* 'Stella de Oro') revealed the presence of 12 of 13 tested phytochemical constituents (Table 5.2). The results also showed that the different tissues and different daylily genotypes contained the different secondary metabolite constituents. The phytochemical compounds in daylily leaf and flower extracts were more diverse than that in root extract. Alkaloids, phenolics, tannins, flavonoids, and terpenoids were presence in all tested samples. Coumarin, glycoside, anthraquinone were not presence in root extract. Saponin was presence in all extracts analyzed excluding leaf and flower extracts of *H. sempervirens.* Also, cardilac glyscoside was not detected in roots and leaves of *H. sempervirens,* and roots of *H. citrina.*

The secondary metabolite constituents in methanol extract of daylily strains observed in present study is in agreement with the studies of Konishi *et al.* (2001), Cichewicz and Nair (2002), Cichewicz *et al*. (2002), Thu *et al.* (2004), Zhang *et al.* (2004), Mao *et al.* (2006), Liu *et al.* (2010), Ogawa and Konishi (2009), Lin *et al.* (2011). The diverse pharmacological properties of these substances have been demonstrated by so many reports.

Alkaloids are characterized by diverse pharmacological effects such as anti-inflammatory, anti-microbial agents, anti-diabetic, effects on cardiovascular system, anti-cancer, antidepressant, and immune effects, etc (Fewell and Roddick, 1993, Morrow *et al*., 2004, Smith *et al*., 2006, El-Tawil *et al*., 2010, Gao and Hu, 2010, Kim *et al.*, 2010, Simera *et al.*, 2010, Adnyana, 2013,

Tomsik *et al.*, 2013). Alkaloids have been used as medicinal productions since so long time ago (Clarke, 1970, Aniszewski, 2007)

Detected compounds of terpenoids group have been demonstrated a wide spectrum of pharmacological and physiological properties, some of those properties have led to a number of terpenoids applied in medicinal applications such as: nutritional supplements, antioxidants, anti-inflammatory, sunlight protection, immune function, antimicrobial, anti-tumor, neurotrophic effects, and fragrant etc. (Elson *et al.*, 1988, Yuan *et al.*, 2010, Miguel, 2011, Espina *et al.*, 2013, Ericson-Neilsen and Kaye, 2014, Justin *et al.*, 2014). Pharmacological activities of glycosides have been showed as anticancer, expectorant, sedative and digestive activities (Fenandec *et al.*, 2006, Newman *et al.*, 2008, Zhou *et al.*, 2013). Anthraquinones possess anti-proliferartive, antioxidant, anti-inflamatory, and anti-arthritic activities (Mishchenko *et al.*, 2007, Kshirsagar *et al.*, 2014). Cardiac glycosides are used as agents against arrhythmic and heart failure to control some heart diseases (Gheorghiade *et al.*, 2006, Hamad *et al.*, 2007). Recently, some pharmacological studies have been revealed the effects in anti-ischemic stroke (Jame *et al.*, 2006) and in anticancer of cardiac glycosides (Lopez *et al.*, 2005, Newman *et al.*, 2007, 2008).

Secondary metabolites of phenolics group that was presence in daylily extracts are also characterized by many valuable bioactivities such as antioxidant, anti-inflamatory, anti-carcinogenic, anticancer, anticonvulsant, anti-allergic, neuroprotective effects, anti-microbial agents (Park *et al.*, 2001, Trouillas *et al.*, 2003, Dai and Mumber, 2010, Gao and Hu, 2010, Venugopala *et al.*, 2013), and may used to treat to some diseases (Pengelly, 2004). The plants containing tannins have been used as remedy to treat diarrhea (Loeb *et al.*, 1989), stomach and duodenal tumors (Fujiki *et al.*, 2012). Beside the same bioactivities of compounds in phenolics

group, recently flavonoids have been thought to play a meaningful role in reducing the risk of cardiovascular disease (Lippi *et al.*, 2010, McCullough *et al*., 2012).

By possessing a wide range of bioactivity, phytochemicals those were detected in the daylily extract may provide a pharmacological explanation for utilizations daylily in traditional medicine to treat a host of diseases and confirm the largely medicinal potential of daylily.

5.3.2. Total phenolic content in daylily extracts

The total phenolic content in methanol extract of roots, leaves, and flowers of three studied daylily strains is shown in Table 5.3. The leaves and flowers contained the significant greater amount of phenolic compounds in comparison with roots. The difference in the total phenolic amount between leaves and flowers depends on each *Hemerocallis* strain. There was no difference of total phenolic content between leaves and flowers observed in *H. sempervirens*, both leaves and flowers contained about 281 mg GAE/g extract. Leaves of *H. citrina* accumulated more phenolic quantity (217.47 mg GAE/g extract) than flowers (105.13 mg GAE/g extract). And in *H.* 'Stella de Oro' cultivar, total phenolic content in flowers (317.13 mg GAE/g extract) was significantly higher than that in leaves (240.47 mg GAE/g extract).

Of three daylily strains, flowers of *H.* 'Stella de Oro' synthesized more phenolic amount (317 mg GAE/g extract) than flowers of *H. sempervirens* (281.80 mg GAE/g extract), but leaves of *H. sempervirens* accumulated more phenolic quantity (281.13 mg GAE/g extract) than leaves of H. 'Stella de Oro' (240.47 mg GAE/g extract). The total phenolic content in both leaves (217.47 mg GAE/g extract) and flowers (105.13 mg GAE/ g extract) of *H. citrina* species was the significantly lowest compared to the two rest strains. No difference in the total phenolic content in root extracts of daylily strains was found (Table 5.3).

5.3.3. Total flavonoid content in daylily extracts

The quantitative analysis of total flavonoids in methanol extract from roots, leaves, and flowers of daylily showed that the total flavonoid content in studied daylily strains ranged from 15.96 to 33.00 mg CE/g extract based on the particular plant parts and specific daylily strains (Table 5.4). As the total phenolic content, the total flavonoid content in roots of daylilies was the significantly lowest in comparison with that in leaves and flowers. The difference of flavonoid content between leaves and flowers varied with the daylily strains. In *H. sempervirens* and *H. citrina*, the leaves accumulated more flavonoids than flowers, but in *H.* 'Stella de Oro' cultivar the content of flavonoids in flowers was higher than that in leaves. Among three daylily strains, *H. citrina* species contained the lowest flavonoid amount (15.96 – 21.52 mg CE/g extract). Flowers of *H.* 'Stella de Oro' showed higher amount of the total flavonoid content than flowers of *H. sempervirens*. No significantly difference in content of the total flavonoids was recorded between roots and leaves of *H. sempervirens* and those of *H.* 'Stella de Oro' (Table 5.4).

There are some reports on content of total phenolic and flavonoid compounds as well as antioxidant activity of daylily extracts published. For examples, Lin *et al.* (2011) showed that in ethanol extracts prepared from dried *H. fulva* flowers, the content of total phenolic and flavonoids is 30.9 mg GAE/g extract and 15.1 mg CE/g extract, respectively. Fu *et al.* (2009) reported that content of catechin in methanol extract prepared from *H. fulva* flower is 64.99-111.53 mg/100 g FW, that is equivalent to 11.48-19.70 mg/g extract by extract yield of *H. sempervirens* flower of 5.66% (Table 5.1). Thus, flavonoid content in daylily extract in present study is in agreement with results cited above. But the total phenolic content (281.80 mg GAE/g extract) is higher as about nine folds as results in report of Lin *et al*. (2011). This fact could be explained by the samples analyzed by Lin *et al.* (2011) is dried. It is assumed that some phenolic structures were lost in dry processing.

5.3.4. Antioxidant capacity of daylily extracts in DPPH radical scavenging assay

The DPPH radical scavenging ability of methanol daylily extracts is expressed by efficient concentration that reduced 50% of the initial DPPH absorbance (EC_{50}). The EC_{50} values of daylily extracts in this study were shown in Table 5.5 and 5.6. The lower value of EC_{50} reflects the better ability of DPPH scavenging, equivalent to the stronger antioxidant activity. In DPPH assay, daylily extracts exhibited corresponding EC_{50} values from 246.98 – 1045.80 μg/mL. Of nine extracts prepared from roots, leaves, and flowers of three studied daylily strains: *H. sempervirens*, *H. citrina*, and *H.* 'Stella de Oro', EC_{50} value of Stella de Oro flower extract was the lowest, and that of *H. sempervirens* root extract was the highest. The significant difference of antioxidant ability of three daylily strains was observed. The roots of Stella de Oro showed the strongest antioxidant activity, next by roots of *H. citrina*, and the weakest antioxidant activity belonged to roots of *H. sempervirens*. The extract from leaves of *H. sempervirens* exhibited the best ability in DPPH free radical scavenging with the lowest value of EC_{50} (284.56 μg/mL), followed by leaves of *H. citrina* with the EC_{50} value of 344.38 μg/mL, and leaves of *H* 'Stella de Oro' possessed the lowest DPPH scavenging ability with the highest EC_{50} value (373.37 μg/mL). The highest EC_{50} value in DPPH assay (677.78 μg/mL) indicated the lowest antioxidant activity of *H. citrina* flowers. Vice versa, the lowest EC_{50} value of *H.* Stella de Oro flowers (246.98 μg/mL) showed its strongest DPPH radical scavenging ability. *H. sempervirens* flowers exhibited the middle antioxidant activity of the other strains with EC_{50} value of 291.98 μg/mL (Table 5.5).

Among methanol extracts prepared from different plant parts of daylily, root extracts presented the highest EC_{50} values in DPPH assay, equivalent to the lowest radical scavenging ability. The difference in antioxidant activity between leaves and flowers was not ruled for all

three daylily strains, leaves of *H. citrina* were better than flowers, but flowers of *H. '*Stella de Oro' were better leaves, and no difference was observed between leaves and flowers of *H. sempervirens* in antioxidant activity (Table 5.6).

Also, data in Table5.5 and 5.6 showed the anitioxidant acivity of three standard antioxidants. Of them, galic acid indicated the lowest antioxidant ability by the highest EC_{50} value (60.20 μg/mL), followed by catechin with EC_{50} value of 46.63μg/mL, and BHT exhibited the strongest antioxidant capacity by the lowest value of EC_{50} (40.61 μg/mL). Based on data of total phenolic and total flavonoid content of daylily presented in Table 5.3 and 5.4, EC_{50} values in DPPH assay of daylily extracts were 246.98 – 1045.80 μg/mL, containing 78.33 – 81.36 μg galic acid /mL, and 8.15 – 18.82 μg catechin /mL. This catechin amount was lower than amount of standard catechin in EC_{50} value (46.63 μg/mL). This difference is assumed that not only catechin but also other phenolic structures in daylily extracts contributed to DPPH scavenging activity. On the contrary, galic acid content in EC_{50} values for DPPH assay of daylily extracts was much higher than that of standard galic (60.20 μg/mL). It is thought that daylily extracts could contain any compounds, those inhibited DPPH scavenging activity.

Results of present study is in agreement with the result of Liu *et al.* (2010), in which EC_{50} values in DPPH scavenging of methanol extract of *H. fulva* flowers ranges from 500 – 630 μg/mL and to reduce 95% of DPPH radicals necessary concentration of *H. fulva* flower extract shoud be 2,375 μg/mL. And EC_{50} value in DPPH assay of this study is higher so much than that of Fu *et al.* (2009) studies, where it is presented that, at concentration of 150 μg/mL daylily flower extract reduced 63.28 – 94.62 % of DPPH radicals. It is assumed that the different strains of daylily collected from different place could contain different constituents and content of antioxidant compounds led in the different effect in DPPH radical scavenging.

5.3.5. Relationship between total phenolic and total flavonoid content and DPPH radical scavenging activity of daylily extracts

Relationship between the content of total phenolics and total flavonoids and the EC_{50} values in DPPH assay was a negative correlation, observed by values of the high correlation coefficient (R^2) (0.89 – 0.99) (with total phenolic content) and (0.60 – 0.98) (with total flavonoid content). This finding is equivalent to have a positive relationship between the content of total phenolics and total flavonoids and antioxidant activity of daylily extracts (Fig. 5.2 and 5.3). The results suggested that both phenolic and flavonoid compounds contributed significantly to antioxidant capacity of daylily.

Many natural products have antioxidant activity. Of which, phenolic compounds including flavonoids is one of the most well-known groups. And the levels of phenolic and flavonoid content have been shown to exhibit antioxidant capacity of medicinal plants (Robards *et al.*, 1999). Many studied found that there is a positive correlation between phenolic content and antioxidant capacity of medicinal plants (Velioglu *et al.*, 1998, Deighton *et al.*, 2000, Djeridane *et al.*, 2006). Similarly, Fu *et al.* (2009) observed a line near correlation between catechin content and antioxidant activity of daylily flower extract and indicated that catechin could be a main compound responsible for antioxidant property of daylily flower extract. Que *et al.* (2007) demonstrated that rutin, catechin, and gallic acid identified in daylily flowers were highly correlated with the antioxidant activities

The antioxidant activity of phenolic compounds in traditionally medicinal plants has been focused by many researches. Phenolics can take part in preventing the body cells from injuries by hydrogen peroxide, protecting human cells from damage of unsaturated fatty acids by lipid peroxides and reducing free radicals (Sroka and Cisowski, 2003). The antioxidant capacities of

these compounds have been thought to link with the lower occurrence and mortality rates of several human diseases.

Table 5.1 Extraction yield of roots, leaves and flowers of three *Hemerocallis* strains

Daylily strains	Samples	Fresh materials (g)	Extract gummy (g)	Extract yield (%)
H. sempervirens	Roots	152.78	24.22	15.85
	Leaves	99.46	5.53	5.56
	Flowers	50.84	2.88	5.66
H. citrina	Roots	142.24	15.11	10.62
	Leaves	101.28	9.98	9.85
	Flowers	52.40	3.54	6.76
H. 'Stella de Oro'	Roots	84.40	6.93	8.21
	Leaves	51.20	3.90	7.62
	Flowers	40.80	2.82	6.91

Table 5.2 Secondary metabolites screening of extracts from the daylily roots, leaves, and flowers

Secondary metabolites	*H. sempervirens*			*H. citrina*			*H.* 'Stella de Oro'		
	Roots	Leaves	Flowers	Roots	Leaves	Flowers	Roots	Leaves	Flowers
Alkaloids group									
Alkaloids	+	+	+	+	+	+	+	+	+
Terpenoids group									
Terpenoids	+	+	+	+	+	+	+	+	+
Steroids	-	-	+	-	-	-	-	-	+
Glycosides	-	+	+	-	+	+	-	+	+
Cardilac glycosides	-	-	+	-	+	+	+	+	+
Anthraquinone	-	+	+	-	+	+	-	+	+
Saponin	+	-	-	+	+	+	+	+	+
Phenolics group									
Phenolics	+	+	+	+	+	+	+	+	+
Tannin	+	+	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+	+	+
Anthocyanines	+	-	-	+	-	-	+	-	-
Leucoanthocyanins	-	-	-	-	-	-	-	-	-
Coumarin	-	+	+	-	+	+	-	+	+

Notes: +: Detected compounds, -: Not detected compounds.

Table 5.3 Total phenolic content as gallic acid equivalent in methanol extracts of three selected daylilies

Daylily strains	Total phenolic content as gallic acid equivalent (mg GAE/g extract)		
	Roots	Leaves	Flower
H. sempervirens	77.80 ± 3.06 Ab	281.13 ± 3.71 Aa	281.80 ± 3.46 Ba
H. citrina	80.47 ± 4.81 Ac	217.47 ± 4.10 Ca	105.13 ± 4.06 Cb
H. 'Stella de Oro'	86.80 ±3.21 Ac	240.47 ± 6.57 Bb	317.13 ±2.91 Aa

Notes: Data are presented as mean ± SE values (n=3). Data in the same column followed by the same upper-letters are not significantly different by LSD ($p<0.05$). And in the same row, data followed by the same lower-letters are not significantly different by LSD ($P<0.05$).

Table 5.4 Total flavonoid content as catechin equivalent in methanol extracts of three selected daylilies

Daylily strains	Total flavonoid content as catechin equivalent (mg CE/g extract)		
	Roots	Leaves	Flower
H. sempervirens	18.00 ± 0.85 Ac	27.81 ± 2.14 Aa	22.81 ± 0.67 Bb
H. citrina	15.96 ± 0.19 Bc	21.52 ± 0.81 Ba	18.74 ± 0.81 Cb
H. 'Stella de Oro'	18.74 ± 0.19 Ac	29.67 ± 0.64 Ab	33.00 ± 0.32 Aa

Notes: Data are presented as mean ± SE values (n=3). Data in the same column followed by the same upper-letters are not significantly different by LSD ($p<0.05$). And in the same row, data followed by the same lower-letters are not significantly different by LSD ($P<5\%$).

Table 5.5 Antioxidant activity effect (EC_{50}) of methanol extracts of some daylily strains and standard antioxidants in DPPH radical scavenging assay

Hemerocallis strains and standard antioxidants	EC_{50} (μg/mL) in DPPH assay		
	Roots	Leaves	Flower
Hemerocallis strains			
H. sempervirens	1045.80 ± 3.52 a	284.56 ± 2.51 c	291.98 ± 4.88 b
H. citrina	967.79 ± 10.99 b	344.38 ± 15.15 b	677.78 ± 15.42 a
H. 'Stella de Oro'	888.04 ± 9.36 c	373.37 ± 10.71 a	246.98 ± 7.44 c
Standard antioxidants			
BHT	40.61 ± 0.33 e	40.61 ± 0.33 e	40.61 ± 0.33 e
Catechin	46.63 ± 1.50 e	46.63 ± 1.50 de	46.63 ± 1.50 de
Gallic acid	66.20 ± 1.80 d	66.20 ± 1.80 d	66.20 ± 1.80 d

Notes: Data are presented as mean ± SE values (n=3). Data in the same column followed by the same lower-letters are not significantly different by LSD ($p<0.05$).

Table 5.6 Antioxidant activity effect (EC_{50}) of methanol extract from plant parts of daylily and standard antioxidants in DPPH radical scavenging assay

Plant parts and Standard antioxidants	EC_{50} (μg/mL) in DPPH assay		
	H. sempervirens	*H. citrina*	*H.* 'Stella de Oro'
Plant parts			
Roots	1045.80 ± 3.52 a	967.79 ± 10.99 a	888.04 ± 9.36 a
Leaves	284.56 ± 2.51 b	344.38 ± 15.15 c	373.37 ± 10.71 b
Flowers	291.98 ± 4.88 b	677.78 ± 15.42 b	246.98 ± 7.44 c
Standard antioxidants			
BHT	40.61 ± 0.33 d	40.61 ± 0.33 d	40.61 ± 0.33 e
Catechin	46.63 ± 1.50 d	46.63 ± 1.50 d	46.63 ± 1.50 de
Gallic acid	66.20 ± 1.80 c	66.20 ± 1.80 d	66.20 ± 1.80 d

Notes: Data are presented as mean ± SE values (n=3). Data in the same column followed by the same lower-letters are not significantly different by LSD ($p<0.05$).

Fig. 5.1 Roots, leaves, and flowers of three studied *Hemerocallis* strains.

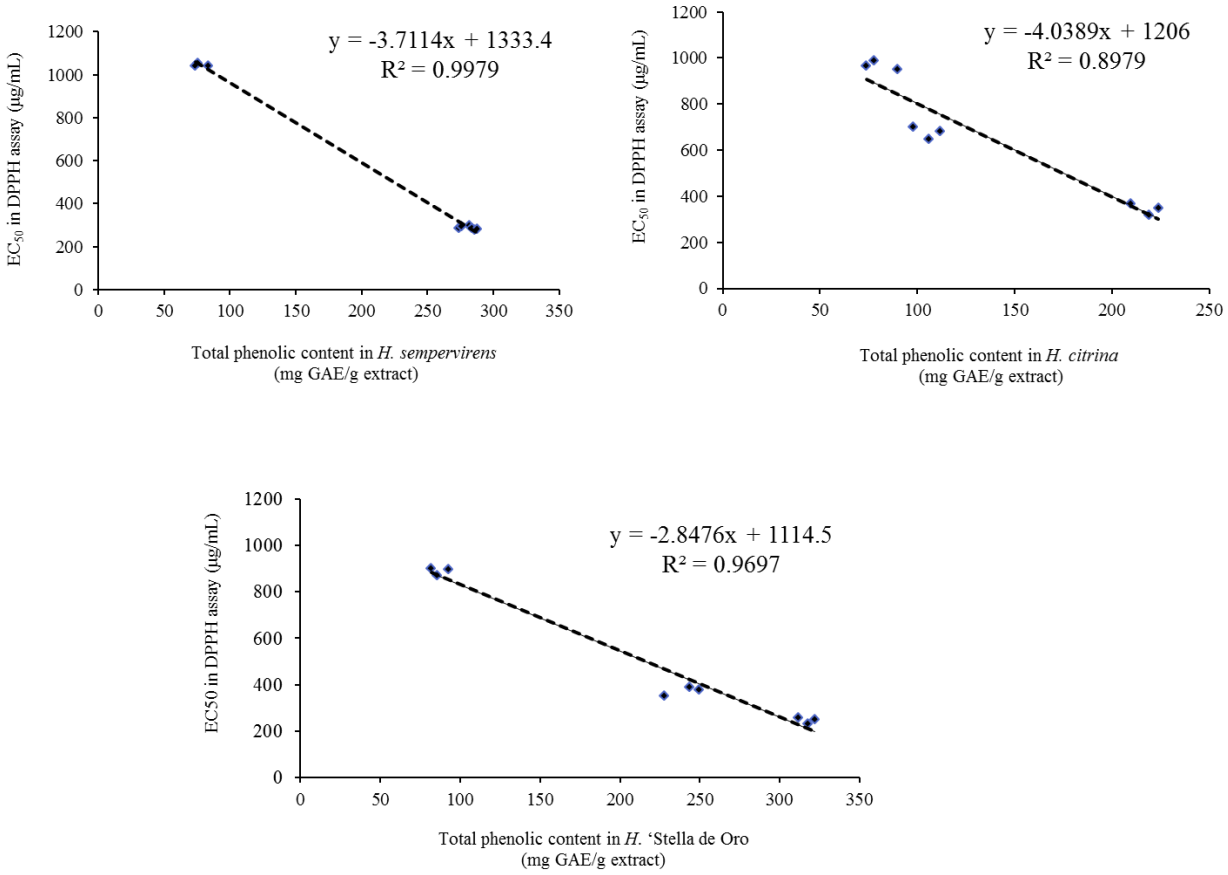

Fig. 5.2 Correlation between total phenolic content and antioxidant activity in DPPH assay of daylily extracts.

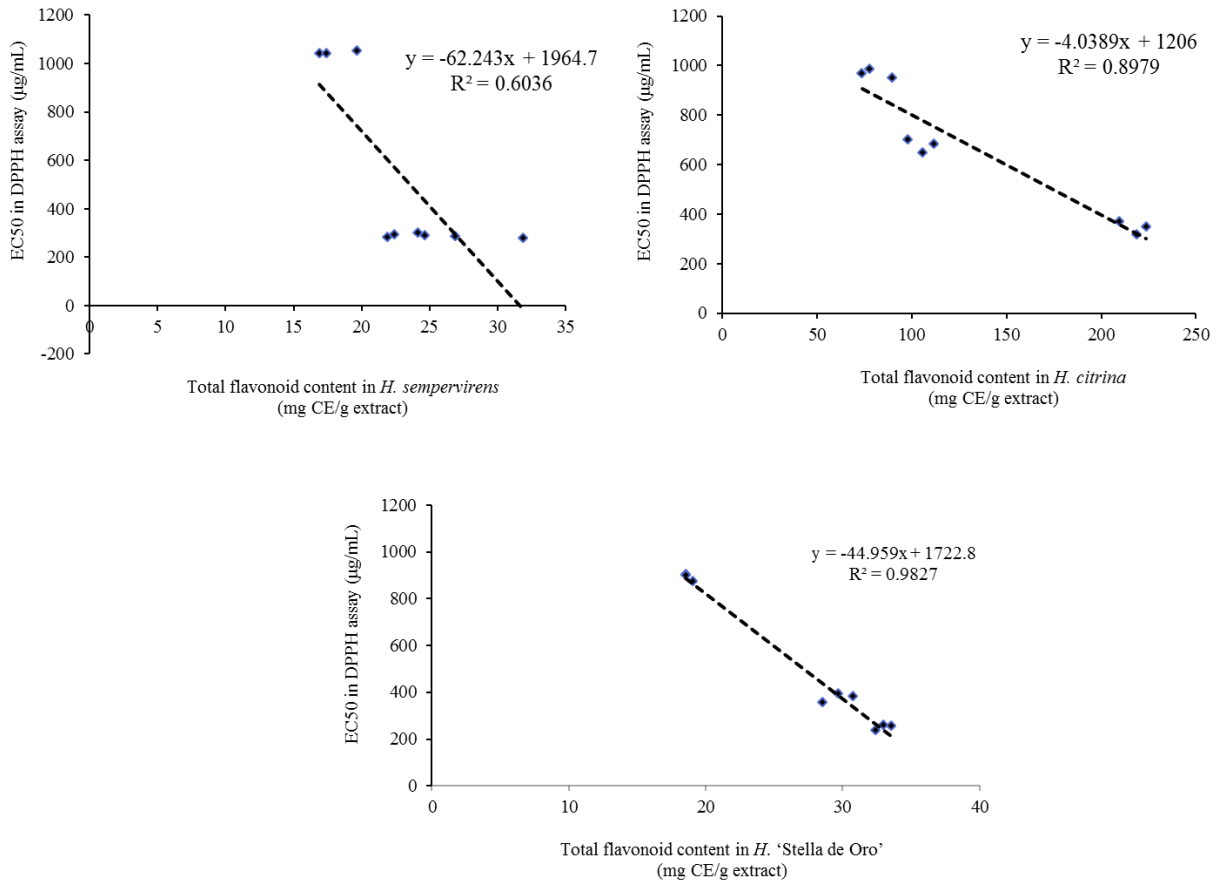

Fig. 5.3 Correlation between total flavonoid content and antioxidant activity in DPPH assay of daylily extracts.

CHAPTER 6

General Conclusions

The present study (1) examined influences of three soil types in Okinawa and N, P, K fertilizations on cultivation of *H. sempervirens* for OPT purpose; (2) evaluated the response of OPT accumulation of *H. sempervirens* to external factors; (3) investigated the variation of OPT concentration in daylily influenced by some internal factors; and (4) analyzed secondary metabolite constituents and antioxidant activities of some daylily strains. And the results of study could be concluded briefly as follows:

1. The growth, yield and OPT accumulation of *H. sempervirens* planted in three soil types in Okinawa (dark-red, gray, and red soils) and of *H. sempervirens* grown in dark-red soil and supplied by eight quantitative combinations of N, P, K at 0.0 – 0.6 g/pot were evaluated. It was found that dark-red soil was the best for plant growth and yield, next by gray soil. The plants in red soil and accumulated the significantly more OPT (2.2 g mg/g FL) than those in gray soil (1.9 mg/g), and similar to plants in dark-red soil (2.2 mg/g), but their growth and yield were lower than those in the other soils. Consequently, dark-red soil is advised to use for cultivation of this plant. The N amount at 0.6 g/pot promoted the leaf yield better, at 0.3 – 0.4 g/pot was better for flower yield, and the combinations of 0.3 – 0.4 g N with 0.3 g P, and K per pot were ideal for OPT accumulation. The results suggested the N, P, K combination at 0.4: 0.4: 0.4 g/pot, respectively is the best for cultivation *H. sempervirens* in dark-red soil to obtain OPT.

2. The OPT concentration in *H. sempervirens* did not vary significantly with condition of waterlogged soil. But it was influenced markedly by the light intensity and the temperature. The

plants grown under 100% and 40% relative light intensity (RLI) produced significantly more OPT than those grown under 75% and 60% RLI. The OPT concentration in plants exposed to 40°C throughout 10 days before harvesting was the highest, followed by that in plants exposed to 15°C. The lowest OPT was observed in plants treated at 10°C. These results suggest that to accumulate high concentrations of OPT, daylily could be grown in both waterlogged and well-watered soils in non-shade or severe shade conditions, and harvested during the vegetative and flowering stages.

In December and January in Okinawa, OPT concentration in *H. sempervirens* was the highest in comparison with the rest months in a year. Thus this is good time for harvesting *H. sempervirens* with OPT purpose. After harvesting, to preserve OPT amount *H. sempervirens* should be stored at room temperature for 2 days, at refrigerator ($0 – 4^{O}C$) for 10 days, and at -20 ^{O}C for 150 days. OPT amount in dried leaves was decreased so much in comparison to that in fresh leaves, so drying is not the way to store H. sempervirens materials to preserve OPT content.

3. All 24 studied daylily strains contained OPT. The internal factors such as genetic diversity and growth stages influenced significantly OPT accumulation of daylily, but ploidy levels (2x and 3x) did not. The OPT accumulated during the vegetative growth and flowering periods was higher than that during the post-flowering period.

4. The phytochemical constituents in daylily were diverse. The total phenolic and total flavonoid content was high and significantly different among three studied daylily strains. The antioxidant capacity in 1,1-diphenyl, 2-picryl hydrazyl radical (DPPH) scavenging assay of extracts from different parts of different daylily strains was significantly different. And content of total phenolics and flavonoids were related positively to the antioxidant capacity of daylily extract.

Beside the obtained results as above, this study would also like to inform methods for determination of OPT concentration in daylily samples. Until now, the information regarding the methods for determination of OPT from daylily has been limited. Based on the little gathered descriptions of OPT such as the UV spectrum of OPT showed only end absorbtion, which contrasted sharply with the strong UV maximum of pinnatanine at 262nm (Grove *et al.*, 1973), or OPT isolated from daylily leaves using recycling HPLC on LC-9 system with GS-310_2 column and some appropriate solvents (Ogawa and Konishi, 2009), method for determination of OPT in daylily extract solution using HPLC system with UV-Vis detector as described in this study was established and tested successfully with the good results indicated.

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