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Effects of several abiotic and biotic factors and plant hormones on growth, morophology, and camptothecin accumulation in Camptotheca acuminata seedlings

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EFFECTS OF SEVERAL ABIOTIC AND BIOTIC FACTORS AND PLANT HORMONES ON GROWTH, MORPHOLOGY, AND CAMPTOTHECIN ACCUMULATION IN *CAMPTOTHECA ACUMINATA* SEEDLINGS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

This dissertation seeks to explore the effects of abiotic factors [nitrogen (N), sodium chloride (NaCl), and ultraviolet (UV) light], biotic factor [acetylsalicylic acid (ASA)], and plant hormones [benzyl adenine (BA) and naphthalene acetic acid (NAA)] on growth, morphology, and secondary metabolite, camptothecin (CPT, an anti-cancer compound) accumulation in *Camptotheca acuminata.* Five experiments were conducted with *C. acuminata* seedlings in a hydroponic system with commercial media and fluorescent lights and at 22-26 °C to investigate the effects of these five factors on height, weight, leaf number, leaf length, root to shoot ratio (RSR), specific leaf weight (SLW), CPT concentrations in leaves and/or roots, and final CPT yield in leaves which were collected in nondestructive harvestings.

N deficiency decreased height, weight, leaf number, leaf length, and leaf chlorophyll concentration in comparison with N supplementation, but increased RSR, SLW, and CPT concentrations in leaves and roots. However, the CPT yield did not increase. NaCl addition reduced height, weight, leaf number, and leaf length, but increased SLW and CPT concentrations in leaves and roots. However, the CPT yield decreased. UV light short-term exposure had no effect on height, weight, leaf number, leaf length, RSR, and SLW, but increased leaf CPT concentration and yield after three weeks. ASA addition reduced plant height, weight, and leaf length, but increased SLW and leaf CPT concentration, and decreased CPT yield. BA application decreased height, but increased RSR and leaf CPT concentration, and had no effect on CPT yield. NAA application decreased height, weight, leaf number, leaf length, SLW, and CPT yield, but increased RSR, and had no effect on leaf CPT concentration.

In conclusion, abiotic and biotic stresses reduced the growth and affected the morphology of *C. acuminata* seedlings in a hydroponic system. Abiotic stresses and biotic stress increased

CPT concentration, but did not increase CPT yield (expect UV). Cytokinin up-regulated CPT concentration but had no effect on CPT yield, whereas auxin did not regulate CPT concentration but decreased CPT yield. A negative relationship between plant growth and CPT concentration existed under the treatments with N, NaCl, or ASA.

GENERAL INTRODUCTION

Background and Significance

Camptotheca acuminata Decaisne (family Nyssaceae) is a deciduous tree species indigenous to southern China with the Chinese name, Tree of Joy. This species was introduced to the USA early in the 1900s in a general plant introduction program. *C. acuminata* trees are commonly raised from seeds (Perdue et al.1970) and cuttings (Liu and Adams 1996). The *in vitro* propagations of *C. acuminata* are also developed (Jain and Nessler 1996, Liu and Li 2001).

Camptothecin (CPT) produced by *C. acuminata* possesses anti-cancer activity that was first identified by Wall et al. (1966) and this is due to its ability to inhibit DNA topoisomerase I (Kjeldsen et al. 1992), an enzyme involved in relaxing super-coiled DNA. Two CPT derivatives, topotecan and irinotecan, were approved by the U.S. Food and Drug Administration in 1996 for the treatment of ovarian and colorectal cancers. The sources of these two compounds rely on the extraction of CPT from raw plant material and chemical modification (such as methylation and/or hydroxylation) of extracted CPT in laboratories to reduce its toxicity and to increase its activity. Hence, production of CPT from plant material remains the only source of these therapeutic compounds. As a result of the discovery of the anti-cancer activity of CPT, *C. acuminata* plantations were established in southern Louisiana to supply plant material for CPT extraction.

Because of the promising clinical uses of CPT, it is important to investigate factors affecting CPT yield in plant material in order to design an effective CPT production system and also extend our understanding of the CPT accumulation mechanism. Here, CPT accumulation includes two aspects: CPT concentration and CPT yield.

CPT is a monoterpene indole alkaloid. As a plant secondary metabolite, it serves to defend the plant against stresses, such as attacks by herbivores and pathogens (Liu and Adams 1996, Taiz and Zerger 1998). CPT accumulates in all parts of *C. acuminata*, having its highest levels in apex and young leaves (Lopez-Meyer and Nessler 1997, Liu et al. 1998, Liu and Adams 1996). A possible reason for a higher CPT level in young leaves is that *C. acuminata* has two defense systems: the chemical defense system and the physical defense system. Young leaves have less physical defense, such as cutin, suberin, and wax, so they employ the chemical defense system which produces a higher concentration of CPT. However, the exact site of CPT biosynthesis is still unknown (Lopez-Meyer et al. 1994), and there is no report on translocation of CPT, a water insoluble compound, within *C. acuminata.*

Many factors were found to affect the accumulation of CPT in *C. acuminata.* CPT concentrations varied significantly with leaf age and tree age, where higher CPT concentrations were found in young leaves and young trees (Liu et al. 1998). Shading of whole seedlings caused higher CPT concentration in leaves and lower CPT concentration in roots (Liu et al.1997). Drought in a field study increased CPT concentration in leaves (Liu 2000). Nitrogen-phosphorus-potassium fertilizers provided to *C. acuminata* seedlings in a field plot either decreased or had no effect on CPT concentration (Liu et al.1999). Methyl jasmonic acid and yeast extract treatments on leaf discs punched from *C. acuminata* seedlings promoted the mRNA expression of tryptophan decarboxylase (TDC), a key enzyme involved in CPT biosynthesis (Lopez-Meyer and Nessler 1997). Similarly, methyl jasmonic acid and yeast extract treatments on *C. acuminata* cell suspension cultures increased CPT accumulation (Song et al.1998).

Similar to CPT, the accumulations of other plant secondary metabolites are also affected by many factors, such as abiotic factors (environmental features, such as climate and edaphic

factors, that do not derive directly from the presence of other organisms), biotic factors (derived from organisms), and plant hormones (Taiz and Zerger 1998, Ouelhazi et al.1994). Meanwhile, plant growth and morphology are also affected by these factors, and plant growth and morphology may relate to plant secondary metabolite accumulation (Larson et al.1990).

This research explored the effects of more factors on plant growth, morphology, CPT accumulation in *C. acuminata*, and defined the relationships between plant growth, morphology and CPT accumulation. The results enabled us to extend our knowledge of CPT accumulation and to use suitable manipulation on specific factor(s) for maximizing CPT yield and designing an effective CPT production system.

Rationale and Related Studies

Rationale

In Fig. 0.1, a complete CPT biosynthetic pathway is given. CPT, as a monoterpene indole alkaloid, is derived from the shikimate pathway and mevalonate pathway. Erythrose-4-phosphate produced by the pentose-phosphate pathway and phosphoenolpyruvate produced by glycolysis condense into shikimate. Shikimate then converts into chorismate. At the same time, inorganic nitrogen (N) ion, NO_3^-, absorbed by plant roots and translocated into leaves, converts into NO_2^- by nitrate reductase and then NH_4^+ by nitrite reductase. NH_4^+ is assimilated into glutamine by glutamine synthetase. Glutamine then transfers an amino group to chorismate by aminotransferase, and tryptophan is formed. Tryptophan becomes tryptamine catalyzed by tryptophan decarboxylase (TDC, coded by *tdc* gene). On the other hand, several acetyl CoAs produced from tricarboxylic acid cycle condense into hydroxyl-methyl-glutaryl CoA (HMG-CoA), which converts into mevalonate by HMG-CoA reductase (coded by *hmg* gene). Mevalonate becomes

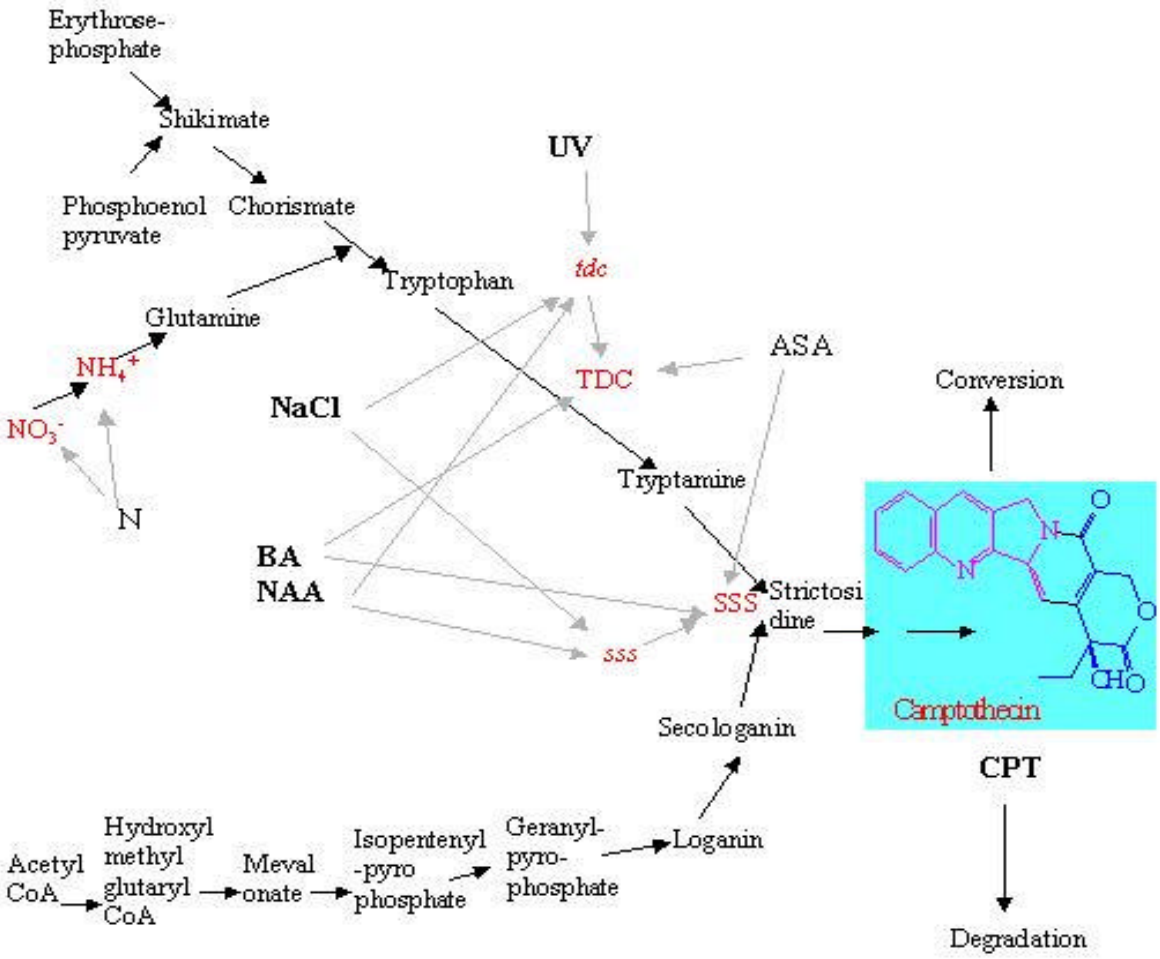

Fig. 0.1. Scheme of CPT biosynthetic pathway and the possible roles of factors investigated in this dissertation

isopentenyl pyrophosphate (IPP) and its isomer, dimethylallyl pyrophosphate (DPP), both of which condense into geranyl pyrophosphate (GPP). This monoterpene then becomes loganin and then secologanin. Secologanin and tryptamine condense into strictosidine by the strictosidine synthase (SSS, coded by *sss* gene). Strictosidine goes several steps and finally becomes CPT.

Abiotic factors, such as N ions (NO_3^- and NH_4^+), as shown in Fig. 0.1, provide the ultimate N source for the biosynthesis of the N-containing compound, CPT. Thus, it is conceivable that adding N to *C. acuminata* might increase CPT biosynthesis and accumulation. NaCl salt stress might stimulate certain stress responsive genes, such as *tdc* and *sss*, based on other studies (Droual et al. 1997). UV light induces accumulation of several monoterpene indole alkaloids as well as the expression of *tdc* gene in *Catharanthus roseus*, and the UV light responsive regions in the promoter of the *tdc* gene in *C. roseus* have been identified (Ouwerkerk et al. 1999). We hypothesize that UV light exposure might stimulate the expression of *tdc* gene and increase CPT accumulation in *C. acuminata.* Biotic factors, especially elicitors [substances produced by invading organisms or damaged plant cells that initiate a complex signaling pathway leading to the activation of plant's defensive response (Taiz and Zerger 1998)], such as acetylsalicylic acid (ASA), might stimulate certain enzymes such as TDC and SSS and promote alkaloid accumulation as shown in other species (Ignotov et al.1996, Godoy and Loyola Vargas 1997). The plant hormone, cytokinin, such as benzyl adenine (BA), might stimulate the activities of enzymes, such as TDC and SSS, to increase the alkaloid biosynthesis as shown in *C. roseus* (Carpin et al.1997). Meanwhile, the plant hormone, auxin, such as naphthalene acetic acid (NAA), might down-regulate the *tdc* and *sss* genes and decrease the alkaloid biosynthesis as found in *C. roseus* plants (Pasquali et al.1992).

To study the effects of the above five factors on CPT yield, we collected data on plant growth, morphology, and CPT concentration, which all related to CPT yield. On the one hand, the effects of these factors on plant growth and morphology were considered because more plant growth will provide more plant biomass, and relatively more shoots or leaves in morphology will give more materials to harvest. On the other hand, CPT concentration in plant material was of concern because higher CPT concentration in plant material will provide an efficient way to increase CPT yield. Finally, understanding the relationships between plant growth, morphology and CPT concentration will extend our knowledge about CPT accumulation and plant secondary metabolism.

To study plant physiology, plant cell culture, tissue culture, root culture, and hydroponic culture were occasionally employed. Our studies were done in a *C. acuminata* hydroponic culture system with well-controlled nutrients, light, temperature, humidity, and well-controlled treatments, such as N treatments, NaCl treatments, ASA treatments, and BA-NAA treatments, in contrast to the various field conditions. The results of these studies are not only useful in a *C. acuminata* hydroponic culture system, but also give us basic knowledge of plant secondary metabolism in *C. acuminata.*

Related Studies

The goals of this section are:

1. To state the advantages and limitations of our hydroponic culture system.
2. To present our preliminary data demonstrating that our new CPT analysis using leaf discs was an effective and accurate tool to minimize CPT variation.
3. To present our preliminary study which demonstrated the relationship between N amendments and CPT concentration in leaves.

4. To present our preliminary data which documented the relationship between UV light and CPT concentration in leaves.
5. To present our supplemental data which demonstrated that punching leaf discs did not affect CPT concentration in leaves.

1. Advantages and limitations of our hydroponic culture system.

In our preliminary study, we developed a well-controlled hydroponic culture system. Plant materials were obtained from our *C. acuminata* tissue culture clone (Liu and Li 2001). The hydroponic culture system had a plastic tray with 6-12 plastic cups containing woody plant medium (WPM, Lloyd and McCown 1981) solution. Seedlings were grown in commercial soil plugs inserted into plastic holders, standing in the solution. The whole tray was covered with transparent cover. This mini-chamber was put underneath fluorescent lights on frames. The temperature was the room temperature (22-26 °C). In this system, we could control plant clone, material uniformity, treatments, light, temperature, and humidity in contrast to the various field conditions. The hydroponic culture system, however, did have some limitations. We could only raise about 40 relatively uniform seedlings for one experiment at a time, and seedlings were only allowed to grow for 3-4 weeks due to nutrient consumption and the limit of the chamber cover. Nevertheless, a 3-4 week culture cycle is standard in plant tissue culture and hydroponic culture due to nutrient consumption (Jain and Nessler 1996, Taiz and Zerger 1998).

2. New sample collection tool and chemical analysis to minimize CPT variation

In this study, CPT levels were measured weekly for 3 weeks. In our preliminary study, we found that it was very hard to do the HPLC analysis and it was very difficult to reduce the variation in CPT concentrations among similar seedlings. At the beginning we had to use about 1 g of dry plant material (or 5 g of fresh plant material), so we used all the leaves on a seedling to

make a sample to conduct HPLC analysis. We found that CPT measurements were limited at the end of the experiment and there was great variation among similar plant material.

We modified the method to minimize the variation. In the new method, we used only 2 leaves per sample for CPT HPLC analysis. A previous study found that leaves at positions 5 and 6 from the apex, which are fully expanded leaves, have relatively more stable CPT concentrations than leaves at positions 1, 2, 3, and 4 (Liu et al. 1998, Fig. 0.2). Thus, we always collected leaves at positions 5 and 6 to make a sample.

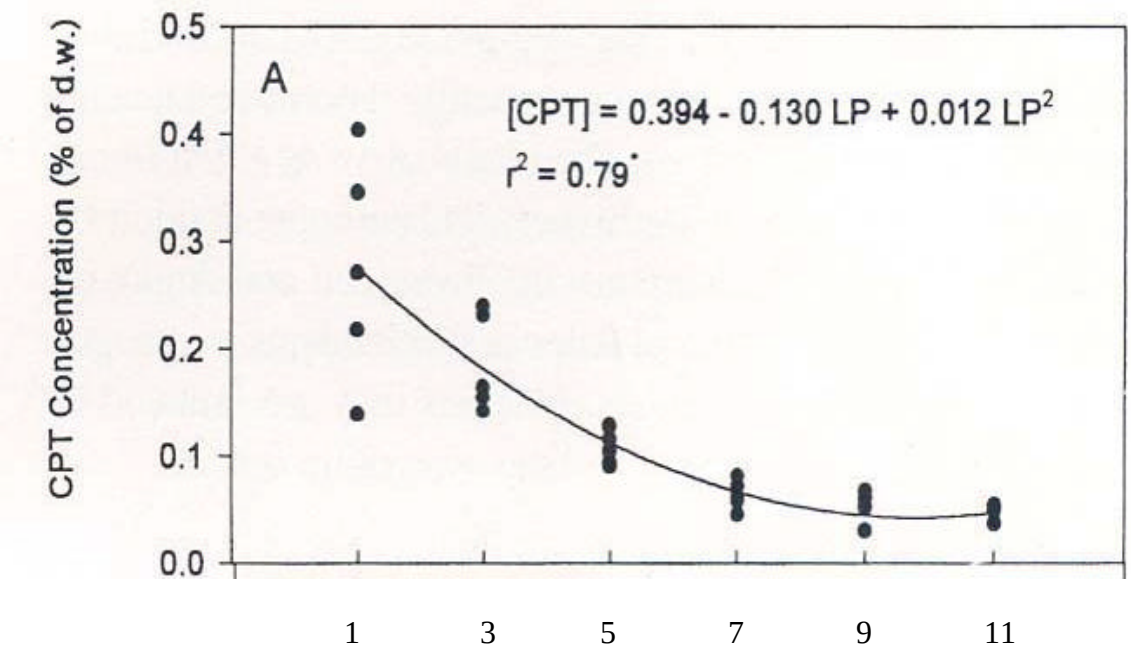

Fig. 0.2. Relationship between leaf position (LP) and CPT concentration. From Leaf 1 toward the base of the branch, every two leaves were grouped and assigned a leaf position in figure. Leaves from three branches per tree were combined for CPT determination (Liu et al. 1998)

However, the damage to seedlings was spectacular, and the variation in CPT concentrations was still relatively large among similar seedlings for our experiment because some leaves were of different sizes and shapes.

Finally, with the help of other plant physiologists, Dr. Liu, Ying Yu and I developed a new method in our laboratory to nondestructively collect and accurately analyze CPT concentrations in plant materials in order to reduce the damage to seedlings and reduce the variation in CPT concentration. In this method, we punched only four 5 mm circular discs from the 5^{th} and 6^{th} leaves (2 discs from each leaf) to make a sample for HPLC analysis. Leaf discs were punched in the middle of the blade and beside the main vein and with exactly the same size (5mm in diameter) to minimize the possible leaf size or location effect. Our pilot study demonstrated the accuracy of our new method as follows (Table 0.1).

Table 0.1. Preliminary study of the accuracy of our new CPT analysis

CPT concentration in leaf (% d.w.)	#s.e. in sampling all leaves	s.e. in sampling two leaves (5-10 cm in length)	s.e. in sampling four leaf discs (5 mm diameter)
0.052	±0.025	±0.01	±0.006

s.e. = standard error

3. Nitrogen study

In our preliminary study, N in a range of 7.5 to 30 milli-mole l^{-1}(mM) in half strength WPM nutrient solution did not produce significant differences in plant height and CPT concentration. In this study we had to use all the leaves on a seedling to make a sample to conduct CPT HPLC analysis. Preliminary data are presented below (Table 0.2). This limited data demonstrated that N enrichments had no effect on plant growth and CPT concentration. This result prompted our study of the effect of N deficiency on plant growth and CPT concentration.

Table 0.2. Preliminary study of nitrogen effect on plant growth and CPT concentration

N treatments	Plant height (cm) #	CPT concentration (% d.w.) #
7.5 mM	12.25±0.6 a	0.052±0.025 a
15 mM	14.30±0.6 a	0.045±0.025 a
22.5 mM	13.61±0.6 a	0.061±0.025 a
30 mM	12.33±0.6 a	0.053±0.025 a

Least square means (lsmeans)±standard error (s.e.) after 4 weeks treatments. Letters represent significant differences in comparisons.

4. UV study

In our preliminary UV study, we amended our HPLC analysis, so we could use 2 leaves on a seedling to make a sample to do CPT analysis. A significant difference in CPT concentrations was found between UV treated and untreated seedlings. Hence, this experiment supported the idea that UV light is an important factor in inducing CPT accumulation.

Table 0.3. Preliminary study of UV light effect on CPT concentration (% d.w.)

Treatments	CPT concentration (week 0)	CPT concentration (week 2)
Control	0.034±0.01a#	0.045±0.01b
UV light	0.031±0.01a	0.088±0.01a

Least square means (lsmeans)±standard error (s.e.) after 4 weeks treatments. Letters represent significant differences in comparisons.

5. Punching study

In our study, we had to punch leaf discs from seedlings to obtain samples. Leaf discs punched from the 5^{th} and 6^{th} leaves served as important samples for CPT analysis in this research. Two leaf discs would be punched from each of the 5^{th} and 6^{th} leaves at weeks 0, 1, 2, and 3 of the treatments using a hole-puncher. Leaf discs were punched from the 5^{th} and 6^{th} leaves because the 5^{th} and 6^{th} leaves had relatively stable CPT concentrations (Liu et al. 1998, Fig. 0.2). About 1%, 2%, and 3% of leaf area was removed at weeks 0, 1, and 2, respectively. We were concerned about the effect of punching on leaf CPT concentration. In a study testing for the induction of alkaloid production, plants were heavily damaged by cutting off 50% of their leaf surface using a pair of scissors, in *Senecio jacobaea* the pyrrolizidine alkaloid concentration in damaged plants decreased within 6-12 hours after damage. Within 24 hours after damage the pyrrolizidine concentration of *Cynoglossum officinale* doubled compared to control values. Indole alkaloid concentration in *Catharanthus roseus* was not increased by the damage (van-Dam Nicole 1993). Wounding resulted in accumulation of mRNAs for three N-containing

compounds, proteinase inhibitors (Hildmann et al. 1992). In two other studies, 7 mm leaf discs were punched from fully expaned *C. acuminata* leaves with a cork borer. Leaf discs were removed at 0h and 24h and frozen in liquid nitrogen for CPT analysis. No differences in *tdc* and *hmg* mRNA expression and CPT concentrations were found, suggesting no effect of punching on CPT accumulation (Lopez-Meyer and Nessler 1997, Maldonado-Mendoza et al. 1997).

The punching of leaves is similar to the effect of insect feeding. But insect feeding on plant leaves not only causes physical damage to the plant but there is also a chemical interaction with the plant through chemicals produced by the insect and/or plant. These chemicals, which are components of insect feeding, are very important in eliciting secondary metabolism (Pearce et al.1991). However, punching leaf discs in our experiment only caused physical damage, and there was no chemical interaction with the plant.

The punching effect on leaf CPT concentration was also studied in our punching experiment. The results for the effect of punching on CPT concentration are shown below. This preliminary data indicated that there was no effect of punching on leaf CPT concentration.

Table 0.4. Supplemental study of punching effect on leaf CPT concentration (% d.w.)

Treatment	CPT concentration (week 0) #	CPT concentration (week 1)	CPT concentration (week 2)	CPT concentration (week 3)
Control	0.060±0.006a	0.064±0.006a	0.047±0.006a	0.045±0.006a
Punching	0.060±0.006a	0.063±0.006a	0.051±0.006a	0.044±0.006a

Least square means (lsmeans)±standard error (s.e.) after 4 weeks treatments. Letters represent significant differences in comparisons.

Overall Objectives and Specific Aims

The overall objectives of this research were to explore more factors affecting CPT yield in *C. acuminata* plant material in order to design an effective CPT production system and also extend our understanding of CPT accumulation and plant secondary metabolism.

The specific aims of this research were to define the effects of several abiotic and biotic factors and plant hormones on *C. acuminata* growth, morphology, CPT concentration, and CPT yield and to find the relationship between plant growth, morphology and CPT concentration. In this study, data on plant growth, morphology, and CPT concentration were gathered prior to and one, two, and three weeks after the treatment with a specific factor. Repeated measures analyses were used to explore the treatment and time effects on plant growth, morphology, CPT concentration, and CPT yield. Covariate analyses of the data were used to identify the relationship between CPT concentration and plant growth, morphology.

The following hypotheses were tested:

1. Abiotic factors, N in media, NaCl in media, and UV light exposure might affect plant growth, morphology, CPT accumulation, and CPT yield. Plant growth, and morphology might relate to CPT accumulation.
2. Biotic factor, ASA might elicit CPT accumulation and affect plant growth, morphology, and CPT yield. Plant growth and morphology might relate to CPT accumulation.
3. Plant hormones, cytokinin and auxin might regulate CPT accumulation. The resulting plant growth and morphology might relate to CPT accumulation.

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CHAPTER 1. EFFECT OF NITROGEN ON GROWTH, MORPHOLOGY, AND CAMPTOTHECIN ACCUMULATION IN *CAMPTOTHECA ACUMINATA* SEEDLINGS

Introduction

It is well established that adequate nutrients, especially nitrogen (N), are required for optimal plant growth. If a nutrient is taken up beyond this concentration (surplus nutrition) it offers no further advantage for growth (Larcher 1995). However, much less is known about the response in plant secondary metabolism. Because an alkaloid, such as camptothecin (CPT) in *Camptotheca acuminata*, is a N-containing compound, excessive N supply beyond the need of plant growth could be presumably shunted into alkaloid biosynthesis based on an N surplus model (Baldwin et al.1993). For example, total alkaloid content in *Tabernaemontana pachysiphon* plants increased with N fertilization (Hoeft et al. 1996). There was a positive relationship between grass yield and alkaloid content in reed canarygrass (Seo and Park 1995).

If N nutrient is taken up below the optimal level (N deficiency), it causes stunting, scleromorphism, and increased root to shoot ratio (RSR) in the plant (Larcher 1995). However, much less is known about the effects of N deficiency on plant secondary metabolism. With a limited amount of a nutrient available in the environment, plant growth and secondary metabolism may compete for the nutrient, and a trade-off between plant growth and secondary metabolite accumulation can occur (Bryant et al. 1983, Hakuiinen et al. 1995). Under N deficiency in a *C. acuminata* growth environment, competition between CPT accumulation and plant growth may exist, i.e. CPT accumulation may be at the cost of reducing plant growth, and vice versa.

It is conceivable that proper manipulation of N availability may obtain a balance between plant growth and CPT accumulation and provide a promising way to increase CPT yield. The

objectives of this study were to examine the effect of N availability on plant growth, morphology, CPT concentration, and CPT yield, and to find the relationships between plant growth, morphology and CPT accumulation.

Materials and Methods

C. acuminata seedlings were propagated in commercial soil plugs from expanding shoot tips of the *ex vitro* plantlets, with each shoot tip bearing three to four leaves (Liu and Li 2001). The plugs were placed in a hydroponic tray filled with 6 liters of half-strength woody plant medium (WPM, Lloyd and McCown 1981) solution adjusted to pH 6.5 and supplemented with 2 mg l^{-1} indole butyric acid and enclosed in a mini-chamber. After two to four weeks, the rooted seedlings with similar height (about 7 cm) and similar leaf numbers (about 6 leaves) were selected and placed into plastic containers containing 100 ml half-strength WPM solution. Seedlings were allowed to acclimate for one week in chambers prior to the experiment.

To assess the effect of N availability on plant growth, morphology, and CPT accumulation, forty seedlings were chosen and divided equally into four groups, with each group assigned to one of four chambers (blocks). In each chamber, seedlings were assigned at random to a 0, 7.5, 15, 22.5 or 30 mM N solution in the following manner. A four-liter full-strength WPM solution free of N was prepared and divided equally into five flasks. N salts made by NH_4NO_3: $Ca(NO_3)_2$ with a molar ratio of 2:1 were then added variably to five flasks to arrive at 0, 7.5, 15, 22.5 or 30 mM N in solution. A 100 ml aliquot of each of these solutions was dispensed into a seedling container. Each N treatment was replicated twice within each of 4 chambers. Seedlings were grown under fluorescent lights providing 40 µmol $m^{-2}s^{-1}$ light intensity with a photoperiod of 16h (16h was used to quicken the plant primary and secondary metabolism as in plant tissue culture (Jain and Nessler 1996) and at the room temperature (22-26 °C).

Plant height, weight, leaf number, and blade length of the third leaf from the apex (leaves longer than 1.5 cm were considered to be the first leaves) were used as indicators of plant growth and were measured at the beginning (week 0) and weeks 1, 2, and 3 of the N treatments. Immediately following these measurements, two circular discs (5mm diameter) were punched (in the middle of the blade and beside the main vein) from each of the 5^{th} and 6^{th} leaves on each seedling, put into a 1.5-ml pre-weighed eppendorf tube, frozen in liquid nitrogen, subsequently freeze-dried, and stored in a refrigerator (2-8 °C) for CPT analysis. A piece of medium-sized root was randomly harvested from each seedling at weeks 2 and 3 of the N treatments and processed in the same way as the leaf discs for subsequent CPT analysis. Roots, leaves, and stems of each seedling were harvested and weighed to obtain the RSR at the end of the 3-week experiment. The RSR and specific leaf weight (SLW, leaf weight per unit leaf area or mg cm^{-2}) obtained from the punched leaf discs were used as indicators of plant morphology.

N ions such as NO_3^-, NH_4^+ can be assimilated and utilized to incorprate into chlorophyll biosynthesis within just one week (Pan and Dong 1996, Taiz and Zerger 1998). In a tissue culture, the maximum consumption of nutrients such as nitrate took place in the first week of the 3-4 week growth cycle (Deliu et al. 1992). In plant tissue culture and hydroponic culture, a period of three to four weeks is usually used as a culture cycle because N and other nutrients will become exhausted and plant growth will stop at the end of this period (Jain and Nesslor 1996, Taiz and Zerger 1998). In this N study, a three-week culture period was used.

The freeze-dried leaf and root samples were ground with a pestle in the eppendorf tubes with 50 µl absolute methanol added first to eliminate the static electricity, and 950 µl methanol was added after the grinding. The samples in locked tubes were extracted for CPT on a rotator for at least 16h at room temperature (22-26°C) in the dark. After sample solids settled to the

bottom of the tube, the supernatant was filtered through a 0.2 µm filter (Whatman Inc. Fairfield, NJ) and transferred into a new 1.5-ml tube. The filtrate solution was dried with nitrogen gas under a hood and reconstituted with 200 µl methanol to achieve a higher concentration of CPT for analysis. Analysis of CPT was performed with a HPLC system (Beckman Instruments, Canton, MA) consisting of a Model 502 autosampler, a Model 125 pump, and a Model 168 photo-diode-array detector as previously described (Liu et al. 1998). CPT concentrations (% d.w.) were obtained on dry weight basis. CPT yields (g) were calculated from the leaf CPT concentrations and the total dry leaf weights [leaves were used in nondestructive harvestings (Lopez-Meyer et al. 1994)] at the end of the experiment.

The leaf discs chlorophyll (Chl) concentration (Chl a + Chl b) in leaf discs was determined following the procedures of Hiscox and Israelstam (1979). The amount of Chl extracted with methanol was determined on a spectrophotometer (Beckman DU-65) at wavelengths of 645 and 663 nm, respectively. Total Chl concentration was calculated as described by Arnon (1949).

Repeated measures analyses were performed for the N and time effects on plant height, weight, leaf number, leaf length, SLW, leaf CPT concentration, root CPT concentration, and leaf Chl concentration. Analyses of variance were performed for the N effect on RSR and CPT yield using the SAS software. Least square means (lsmeans) were obtained and compared using least significant difference by Student's t test. Plant growth, morphological, and chemical data were analyzed systematically using covariate analysis with backward variable selection to find the relationships between plant growth, morphology and CPT concentration and to identify which variables were significantly related to CPT accumulation. In the full model, CPT concentration

in leaves was used as response variable, and plant height, weight, leaf number, leaf length, RSR, and SLW were used as explanatory variables. All tests were regarded as significant at $P \leq 0.05$.

Results

Plant Growth

N availability ranging from 0 to 30 mM had a significant interaction with time on plant height of *C. acuminata* (Table 1.1). There were no differences in plant height among all N treatments at weeks 0 and 1 of the treatments, but there was significantly lower height with the N deficiency (0 mM) in comparison with the N supplement at weeks 2 (except for N at 30 mM) and 3 (Fig. 1.1). However, there were no differences in plant height among the N supplement levels from 7.5 to 30 mM in the media.

There was a significant interaction between N treatments and time on plant weight (Table 1.2). There were no significant differences in plant weight among all treatments at week 0, but there were significant differences at week 3 of the treatments, where the N deficiency produced significantly less weight than all the N supplement levels (except for N at 30 mM) (Fig.1.2).

The effect of N treatments on leaf number depended on time (Table 1.3). There were no differences for all treatments at weeks 0 and 1, but there were differences at weeks 2 and 3 (Fig. 1.3). The lack of N in the media limited significantly leaf number, whereas N supplements induced significantly more leaves compared with N deficiency. However, increasing N concentrations from 7.5 to 30 mM did not result in a corresponding increase in leaf number.

The effect of N on leaf length changed over time (Table 1.4). Leaf lengths were similar at weeks 0, 1, and 2 of the treatments (Fig. 1.4). But there were significant differences at week 3, where leaves on N-deficient seedlings had the least length.

Table 1.1. Repeated measures analysis of nitrogen effect on plant height

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	32	21.25	0.0134
N	4	32	3.21	0.0004
Time	3	32	215.06	<0.0001
N*Time	12	32	5.14	0.0140

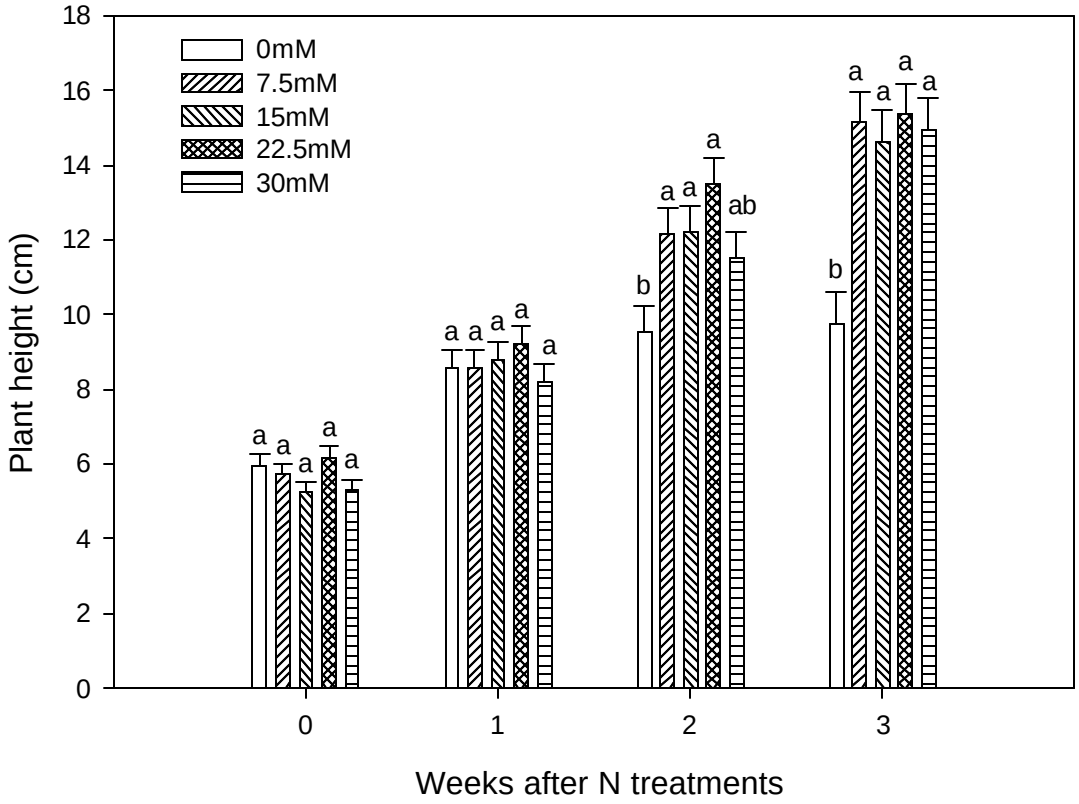

Fig. 1.1. Effect of nitrogen on height of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate significant differences of t tests among the lsmeans at P≤0.05.

Table 1.2. Repeated measures analysis of nitrogen effect on plant weight

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	32	2.72	0.0608
N	4	32	1.86	0.1414
Time	1	32	94.54	<0.0001
N*Time	4	32	2.90	0.0373

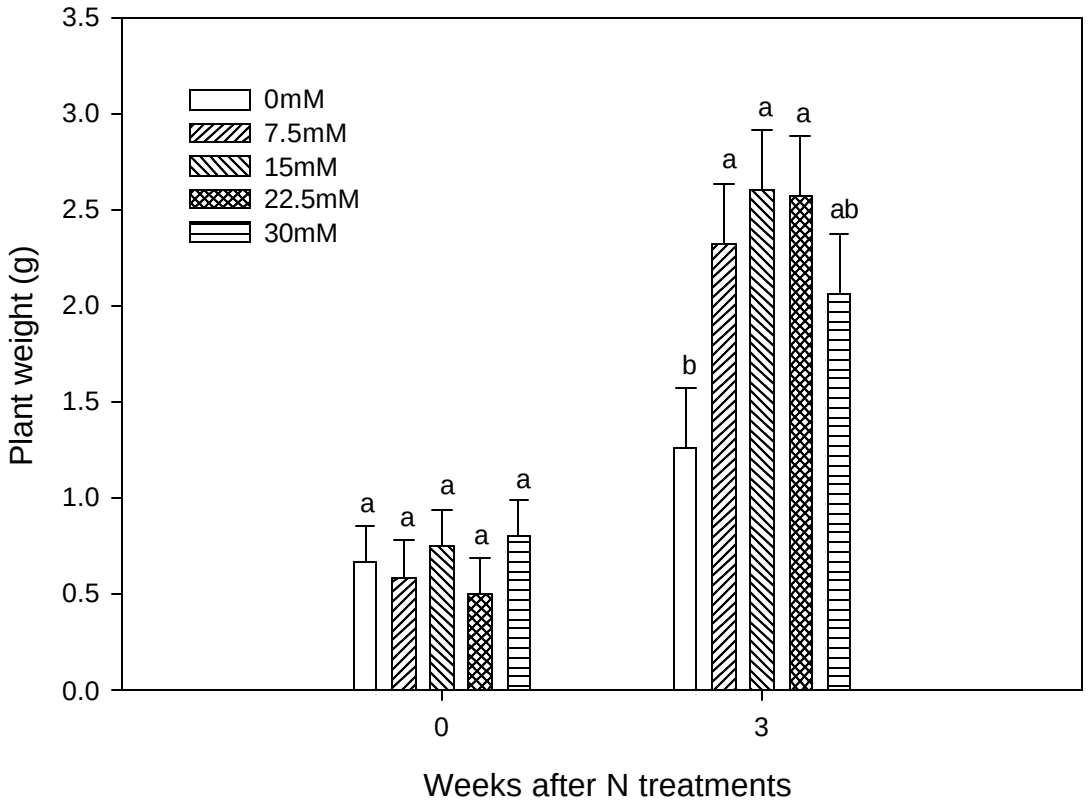

Fig. 1.2. Effect of nitrogen on weight of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant differences of t tests among the lsmeans at P≤0.05.

Fig. 1.3. Repeated measures analysis of nitrogen effect on leaf number

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	32	8.75	0.0002
N	4	32	3.73	0.0134
Time	3	32	229.10	<0.0001
N*Time	12	32	5.28	<0.0001

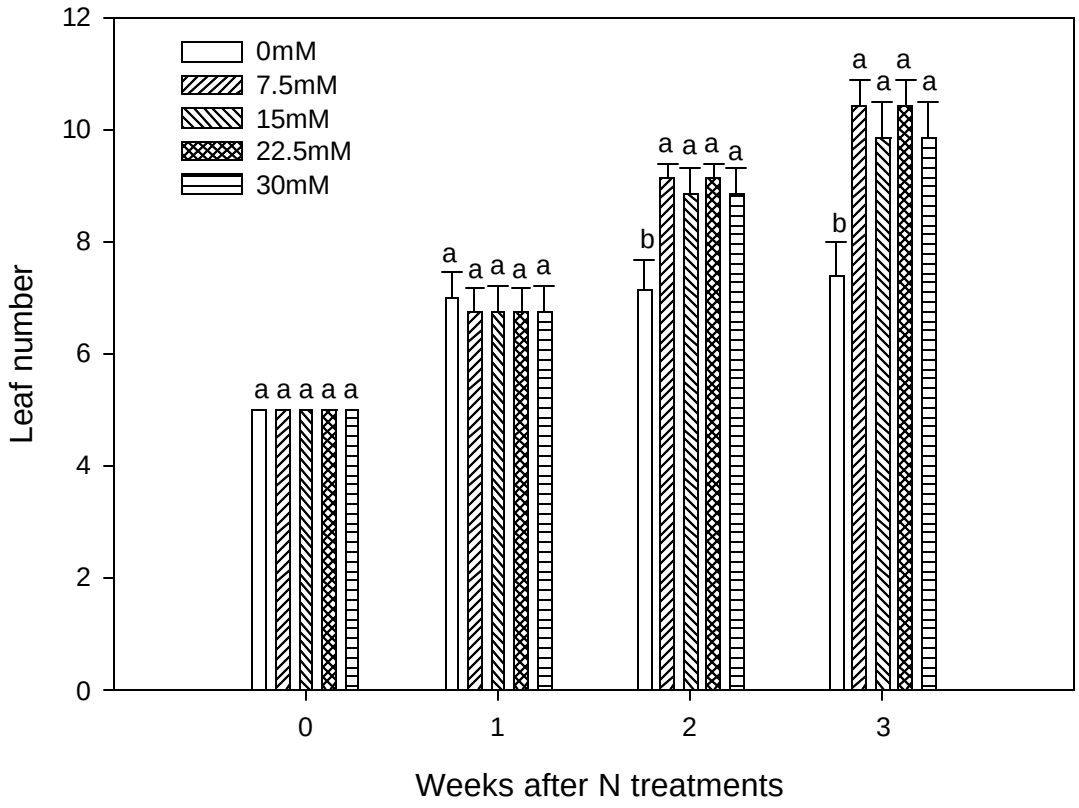

Fig. 1.3. Effect of nitrogen on leaf number of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant differences of t tests among the lsmeans at P≤0.05.

Table 1.4. Repeated measures analysis of nitrogen effect on leaf length

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	32	7.27	0.0008
N	4	32	2.08	0.1062
Time	3	32	74.26	<0.0001
N*Time	12	32	3.18	0.0044

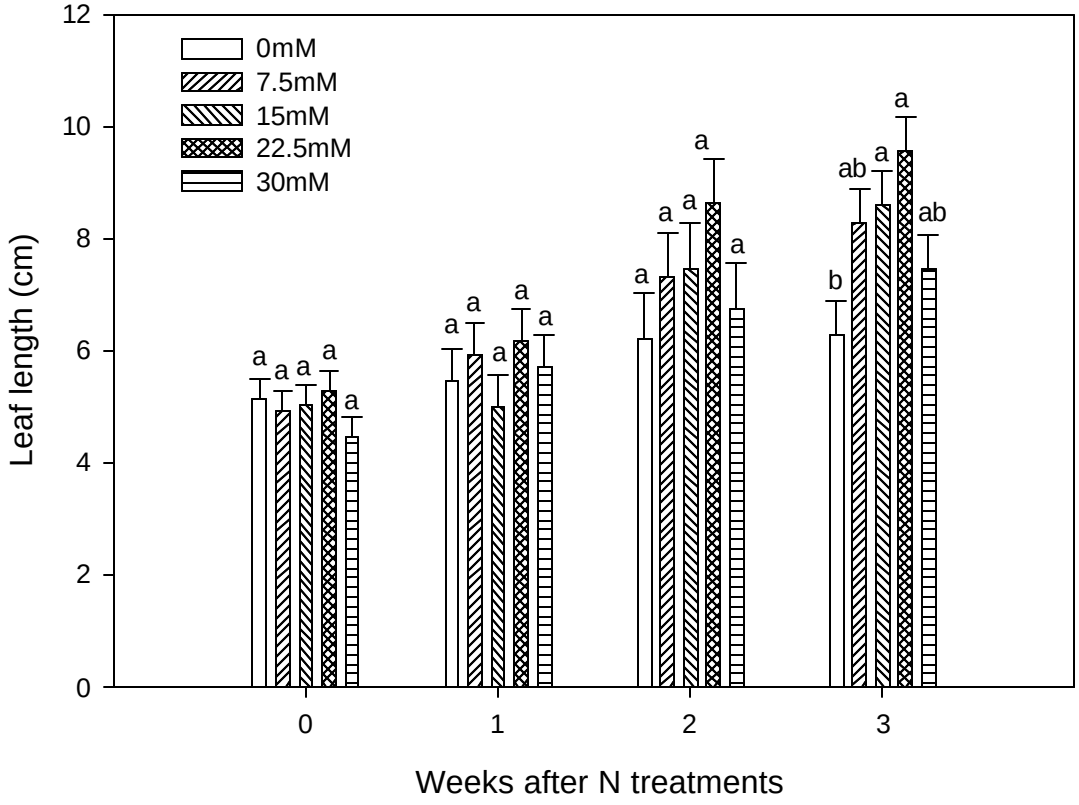

Fig. 1.4. Effect of nitrogen on leaf length of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant differences of t tests among the lsmeans at P≤0.05.

Plant Morphological Changes

N availability significantly affected the RSR (Table 1.5). The N deficiency produced the highest RSR or relatively more roots at week 3 of the treatments, and increasing N concentration resulted in decreased RSR or relatively more shoots (Fig. 1.5).

There was a significant interaction between N treatments and time on SLW (Table 1.6). SLW did not change significantly one week after the N treatments (Fig.1.6). However, there were significant differences in SLW among N treatments two and three weeks after the treatments, where the N deficiency produced the highest SLW compared with the different N supplement levels, whereas N supplements of 7.5 to 30 mM resulted in no change in SLW.

CPT Concentrations in Leaves and Roots and CPT Yield

The effect of N on leaf CPT concentration depended on time (Table 1.7). Prior to N treatments, there were no differences in leaf CPT concentrations among all the treatments, indicating homogeneous experimental materials (Fig. 1.7). Leaf CPT concentrations were also similar among all treatments one and two weeks after the treatments. CPT concentrations in N-deficient leaves were significantly greater than those in N-supplemented leaves three weeks after the treatments.

The effect of N on root CPT concentration also depended on time (Table 1.8). There were no differences in root CPT concentrations two weeks after the treatments (Fig.1.8). Significant differences were observed three weeks after the treatments, where the N deficiency led to higher root CPT concentrations than the N supplement treatments. Similar changes occurred both in root CPT concentrations and in leaf CPT concentrations at week 3, but root CPT concentrations were slightly lower than leaf CPT concentrations.

Table 1.5. Analysis of variance of nitrogen effect on RSR

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	32	0.72	0.5462
N	4	32	10.41	0.0001

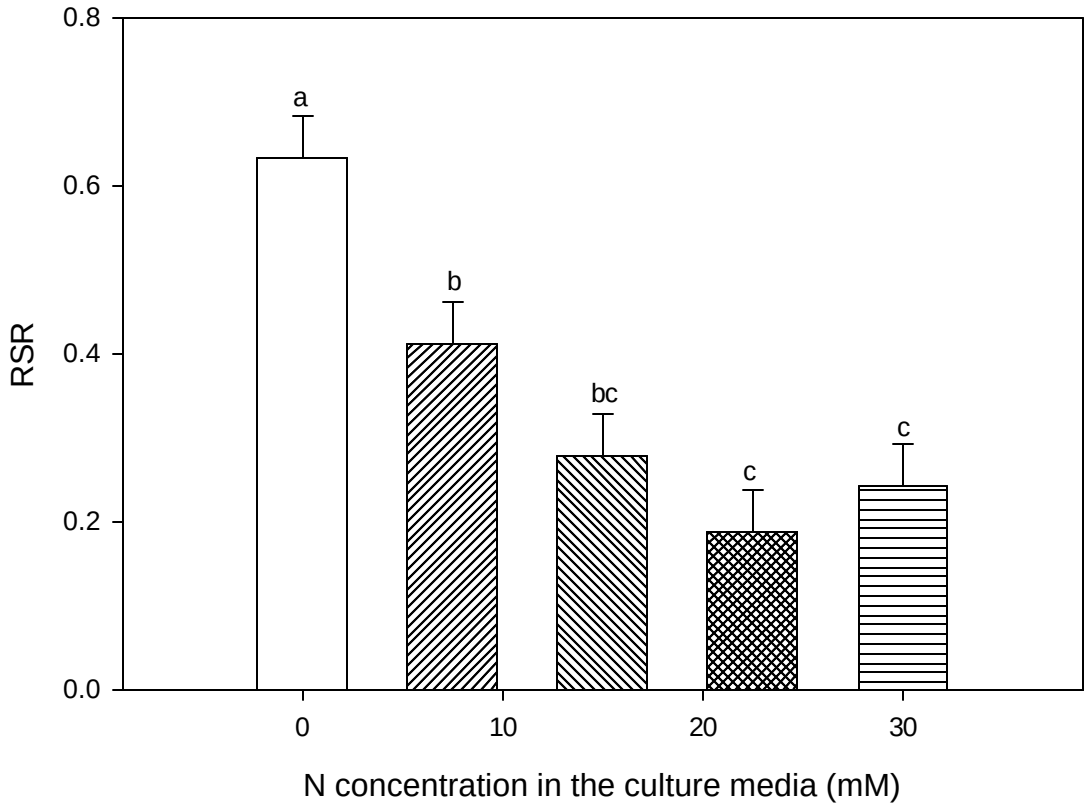

Fig. 1.5. Effect of nitrogen on RSR of *C. acuminata* seedlings in a hydroponic system at week 3 of the treatments. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant differences of t tests among the lsmeans at $P \leq 0.05$.

Table 1.6. Repeated measures analysis of nitrogen effect on SLW

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	32	3.22	0.0355
N	4	32	10.57	0.0001
Time	3	32	45.47	<0.0001
N*Time	12	32	3.43	0.0026

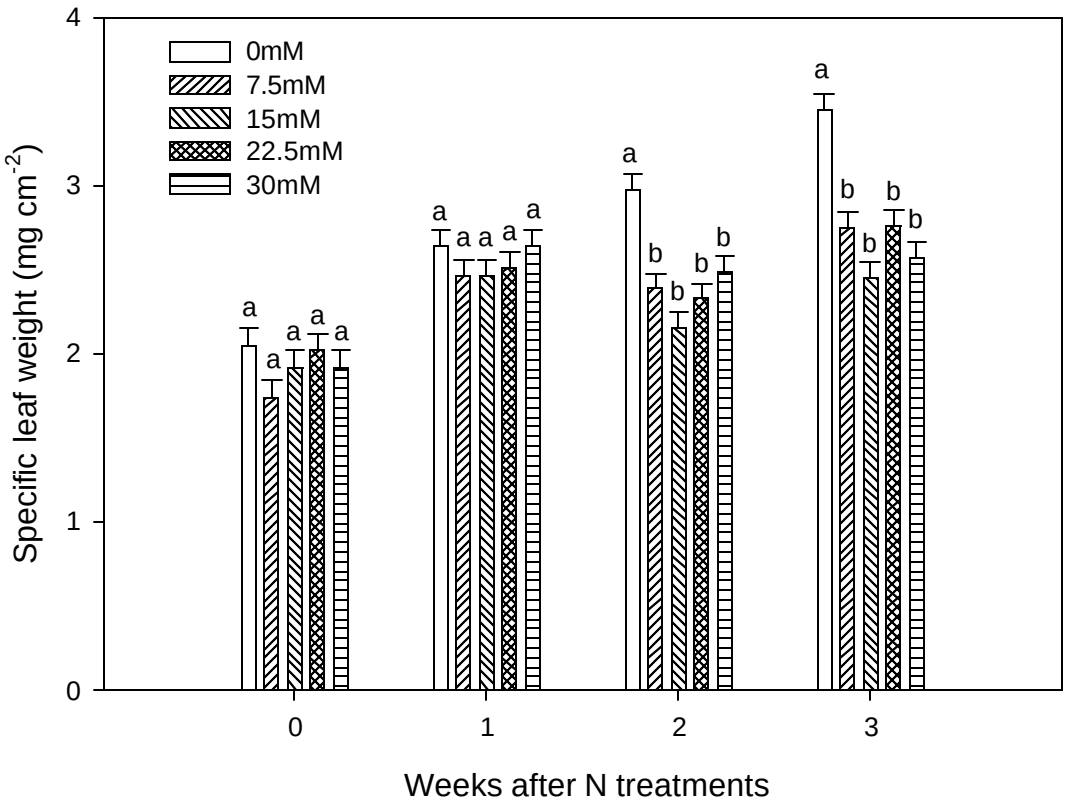

Fig. 1.6. Effects of nitrogen on SLW of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant differences of t tests among the lsmeans at $P \leq 0.05$.

Table 1.7. Repeated measures analysis of nitrogen effect on leaf CPT concentration

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	32	17.41	<0.0001
N	4	32	1.54	0.2143
Time	3	32	28.20	<0.0001
N*Time	12	32	2.81	0.0096

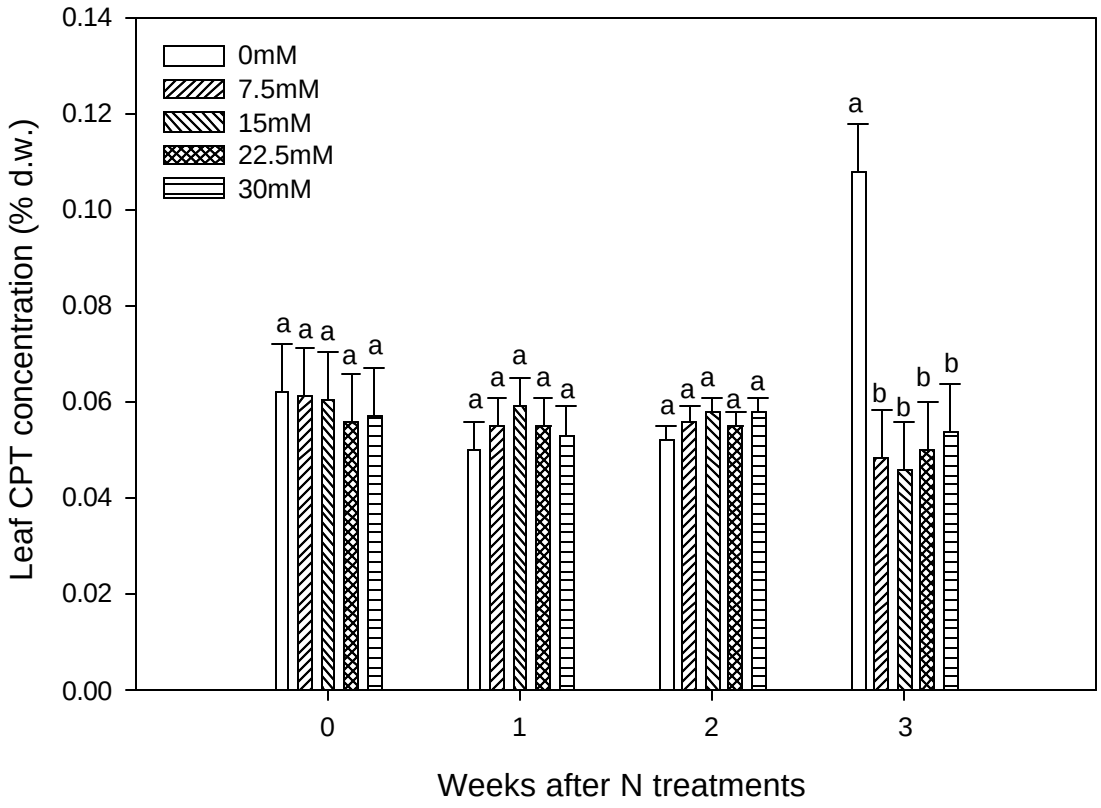

Fig. 1.7. Effect of nitrogen on leaf CPT concentration of *C. acuminata* seedlings in a hydroponic system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant differences of t tests among the lsmeans at P≤0.05.

Table 1.8. Repeated measures analysis of nitrogen effect on root CPT concentration

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	29	4.00	0.0169
N	4	29	3.36	0.0223
Time	1	29	0.97	0.3340
N*Time	4	29	5.22	0.0070

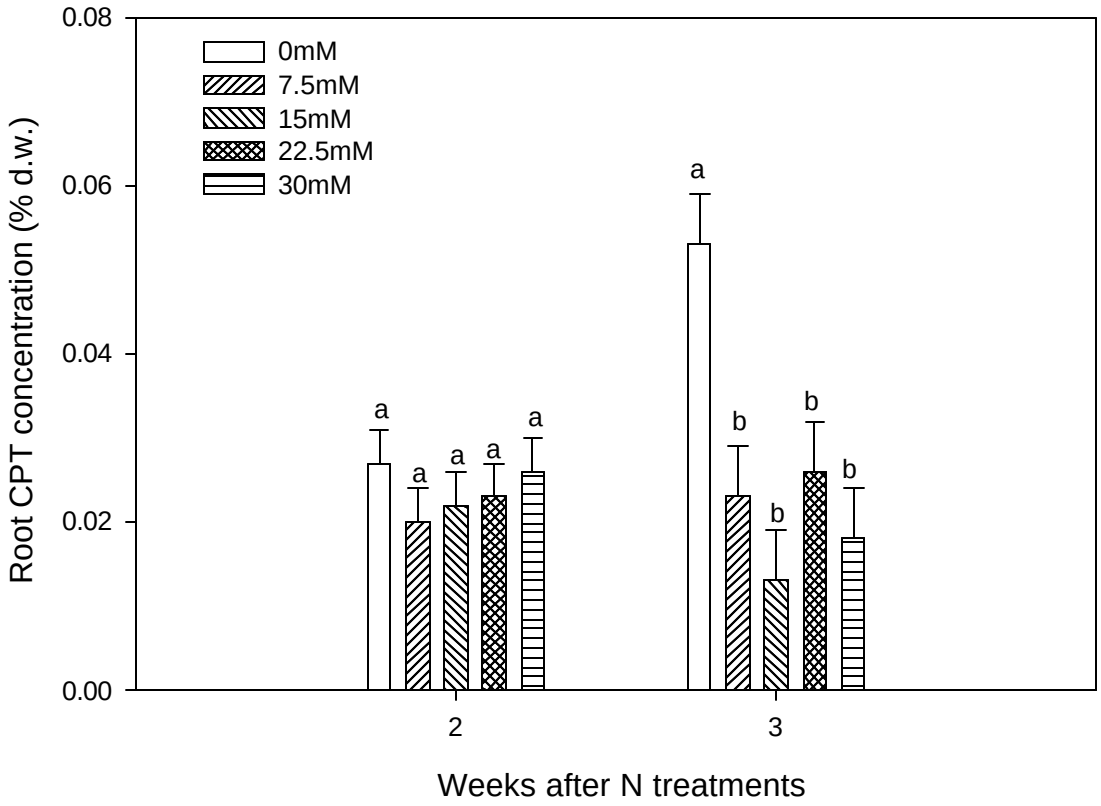

Fig. 1.8. Effect of nitrogen on root CPT concentration of *C. acuminata* seedlings in a hydroponic system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant differences of t tests among the lsmeans at P≤0.05.

N treatments had a significant effect on the final CPT yield (Table 1.9). N addition of 0 to 22.5 mM produced similar CPT yields, whereas N addition of 30 mM produced the lowest CPT yield at the end of the three-week experiment (Fig. 1.9).

Leaf Chlorophyll Concentrations

Leaf Chl concentration was affected significantly by N availability and time (Table 1.10). Chl concentrations were much lower in N-deficient leaves than those in N-supplemented ones two and three weeks after the treatments (Fig. 1.10). However, N supplement in the range of 7.5 and 30 mM produced similar leaf Chl concentrations. Meanwhile, leaf Chl concentrations among all treatments decreased significantly with time from week 2 to week 3.

CPT Concentration and Plant Growth, Morphology

Covariate analysis with backward variable selection was performed (Table 1.11). Plant height, RSR, and Chl concentration were significantly related to CPT concentration. Plant height and leaf Chl concentration were negatively related to CPT concentration, whereas, RSR was positively related to CPT concentration.

Discussion and Conclusions

Plant Growth

N availability as found in this study affected significantly *C. acuminata* growth in plant height, weight, leaf number, and leaf length, as in other species, such as *Catharanthus roseus* (van-Iersel et al. 1998, 1999). Our findings support the general idea that N is an essential nutrient element for plant growth. Furthermore, the full strength WPM basal media in our study provided adequate nutrients for plant growth (Lloyd and McCown 1981) except for the N in question. However, N in the range of 7.5 to 30 mM in the growth media produced no difference in growth of *C. acuminata.* Similarly, in a *Catharanthus roseus* study, where N at 6, 12, 18, and 24 mM

Table 1.9. Analysis of variance of nitrogen effect on CPT yield

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	31	4.50	0.0098
N	4	31	3.16	0.0274

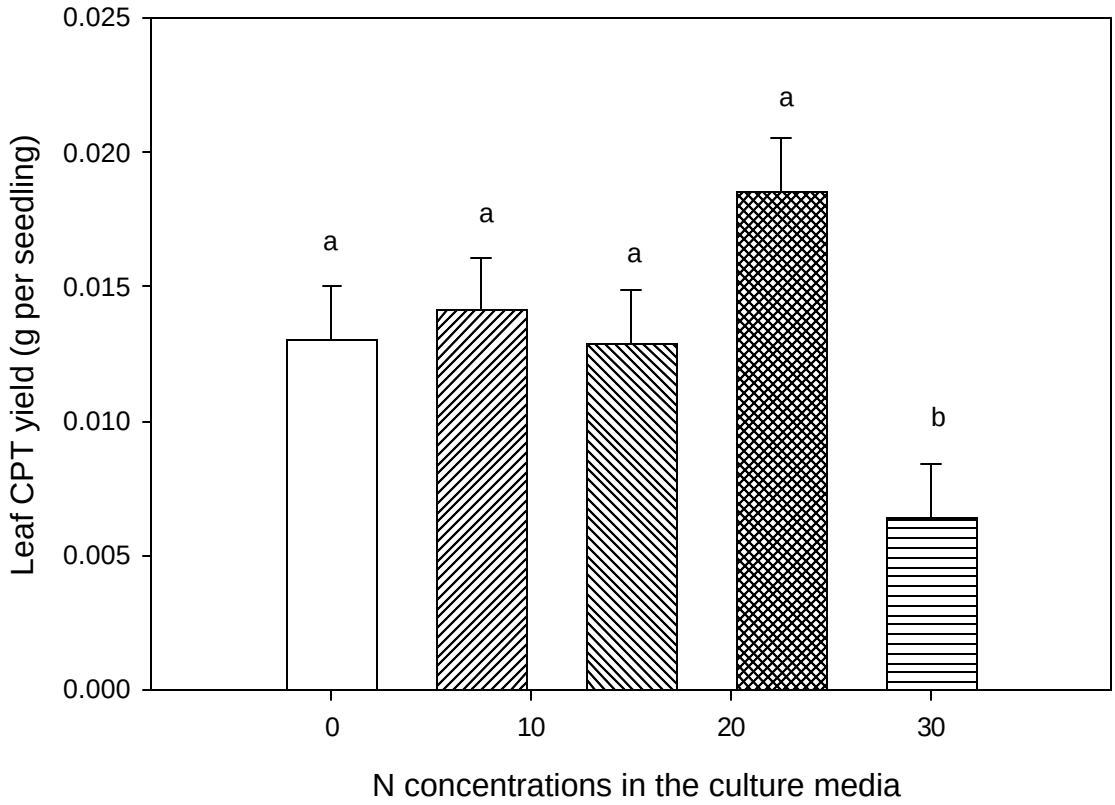

Fig. 1.9. Effect of nitrogen on CPT yield of *C. acuminata* seedlings in a hydroponic system at week 3 of the treatments. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant differences of t tests among the lsmeans at P≤0.05.

Table 1.10. Repeated measures analysis of nitrogen effect on leaf Chl concentration

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	29	3.60	0.0253
N	4	29	41.15	0.0001
Time	1	29	91.90	<0.0001
N*Time	4	29	1.76	0.0966

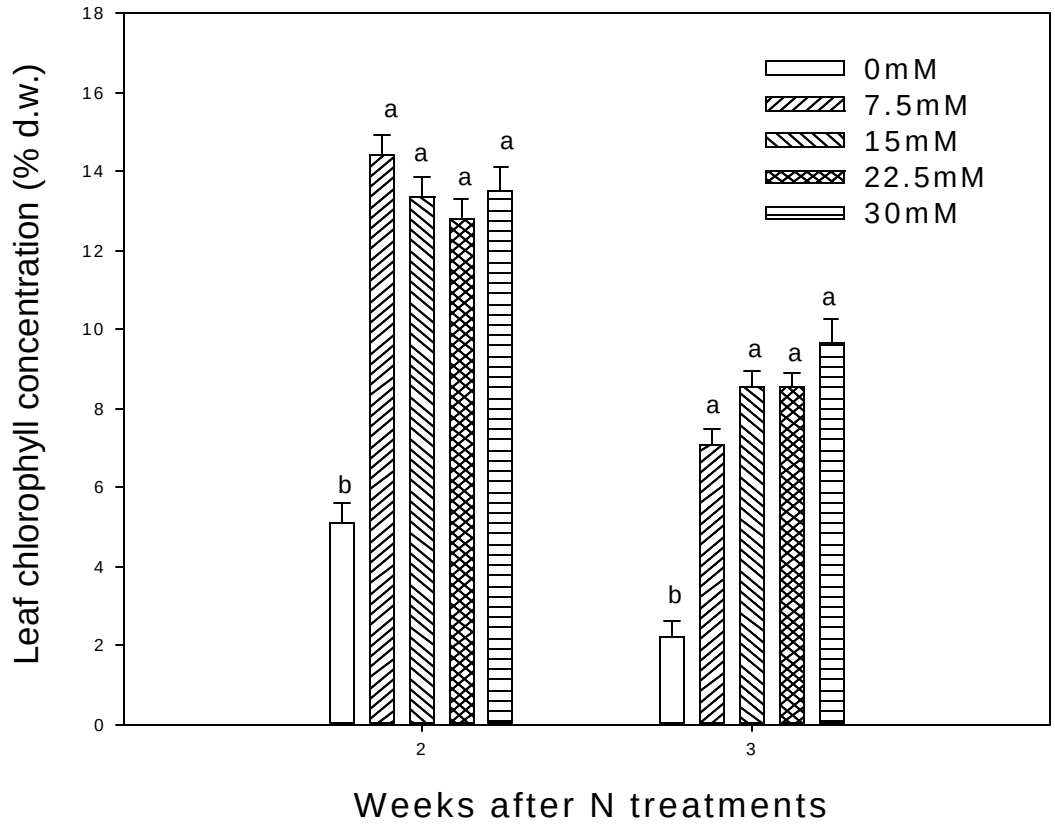

Fig. 1.10. Effect of nitrogen on leaf Chl concentration of *C. acuminata* seedlings in a hydroponic system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant differences of t tests among the lsmeans at P≤0.05.

Table 1.11. Covariate analysis with backward variable selection for N study

Variable	Estimate	Type II SS	F Value	P-value
Intercept	0.00242	0.00003	0.00	0.9466
Height	-0.02072	0.00445	5.81	0.0237
RSR	0.08619	0.00658	8.59	0.0071
Chl	-5.45059	0.00496	6.48	0.0174

produced no difference in growth and biomass (Rho and Andre 1991). In his book, *Physiological Plant Ecology*, Larcher (1995) described this phenomenon in terms of the relationship between the concentration of a mineral and plant growth. With an adequate mineral supply, a plant has optimal growth. Once a plant's mineral requirement has been met, any excess beyond this point seems to bring no additional benefit (surplus nutrition) for growth, and there is a possibility that the surplus nutrient is used instead to promote the plant defense system.

Plant Morphologic Changes

The results of this experiment indicated that N deficiency resulted in significant morphological changes in *C. acuminata* within a period of three weeks, including the higher RSR and SLW (heavier leaves). Conversely, N supplement treatments resulted in lower RSR and SLW (lighter leaves). Rapid increases in roots shortly after N deficiencies were observed in other plant species (van der Werf and Nagel 1996, Nagel et al. 2001). The dramatic change in plant morphology may be a result of adaptation to changing environmental conditions. The increased RSR may be one of the adaptations to nutrient starvation, where more roots are needed to obtain nutrients. The increased SLW may be due to a build up of excess carbohydrates that cannot be used in the synthesis of amino acid or other N-containing cocompounds (Larcher 1995), and the resulting fibrous leaves may be used to create a physical barrier against further herbivore feeding.

CPT Concentration and Possible Mechanism

Since N is a component of the alkaloid, CPT molecule, and N salt is the ultimate N source for CPT biosynthesis, it is conceivable that N deficiency could decrease and N supplementation could enhance CPT biosynthesis from a substrate point of view. This was reported in other species such as reed canarygrass (Seo and Park 1995) and *Uncaria rhynchophylla* (Kawazoe et al. 1993). Our results indicated that CPT concentrations in leaves and roots did not decrease one and two weeks after the N-deficiency treatment. More interestingly, CPT concentrations in leaves and roots increased three weeks after enduring a N deficiency. This increase in CPT concentration might be one of the consequences of stunted growth due to external N shortage. We hypothesize that under the stress of limited N supply, plant adjusts in such a way that secondary metabolism is prioritized over growth. Since there was no source of N in the growth media, the readjustment leading to a higher CPT concentration must have taken place internally. It is possible that the increased CPT molecules had utilized a new N source that was not available when N was not limited, but became available when N was deficient. A possible N source could come from the Chl degradation because Chl concentration declined with time in this study and there was a significant negative relation between leaf CPT concentration and Chl concentration (Table 1.11).

CPT concentrations in leaves and roots did not increase even thought the external N supply was enriched. We hypothesize that when N supply is enriched, more N is utilized for growth rather than for plant secondary metabolism. These results are also supported by a previous field fertilization study with *C. acuminata* (Liu et al. 1999) and a study with *C. roseus* (Tebet et al. 1996). Our data do not support the N surplus model that excessive N beyond the need of plant growth could be shunted into alkaloid biosynthesis. Supporting our findings is a

report on *Nicotiana sylvestris* showing that N in an amount exceeding its growth requirement was not shunted into nicotine biosynthesis (Baldwin et al. 1994).

Our results suggest that N as a substrate is not directly related to the plant secondary metabolism, and increasing this substrate may not necessarily foster the utilization of N in plant secondary metabolism. In other words, CPT accumulation is not merely a process triggered by the availability of N, but rather, depends more on its programmed ontogenic development and environmental stimuli. Lopez-Meyer and Nesslor (1997) and Maldonado-Mendoza et al. (1997) reported the discoveries of two families of genes in *C. acuminata* plant: *tdc*1, *hmg*2, and *hmg*3 expressed for CPT biosynthesis during ontogenic development, and *tdc*2 and *hmg*1expressed for CPT biosynthesis during environmental stresses. The N deficiency for a period of 3 weeks increased leaf CPT concentration by possibly creating an environmental stress through the N deficiency itself. The biosynthesis of defensive metabolites is regulated by the plant, depending on the ability of the plant to acclimate to changes in the physical environment (Lavola and Julkunem Tiitto 1994). For example, in *Nicotine sylvestris*, root alkaloid metabolism was directly influenced by stress, not by N surplus (Baldwin et al. 1993), and nicotine biosynthesis in *N. sylvestris* was not influenced by an exogenous N source (Baldwin et al. 1994). Our results suggest that CPT, like other alkaloids, serves mainly as a plant defensive chemical and its accumulation is more sensitive to environmental stimuli (Liu 2000) and development (Liu et al.1998) than to substrate availability.

CPT Yield

N deficiency produced similar CPT yield as N addition of 7.5 to 22.5 mM, because final CPT yield in leaves depended on two factors: plant biomass and CPT concentration. Although N deficiency had the highest CPT concentration, it also had the lowest plant biomass and so it did

not produce the highest CPT yield. On the other hand, the highest N addition produced the lowest CPT yield, because it had a similar CPT concentration as other N supplementation but it also had relatively less biomass.

CPT Concentration and Plant Growth, Morphology

The significant negative relationship between plant height and CPT concentration, between leaf Chl concentration and CPT concentration may suggest that there is a trade-off between plant growth and CPT accumulation. A negative relationship between plant growth and alkaloid production were also shown in field-grown *C. roseus* (Rajeswara and Singh 1990), and in ragwort, where plants with higher pyrrolizidine alkaloid concentration grew more slowly than those with lower pyrrolizidine concentration under light-limiting conditions (Vrieling and van Wijk 1994). The reason for this trade-off may be that an economic reallocation of resources and energy occurs under stress conditions (van der Werf and Nagel 1996).

Our results showed a significant positive relationship between CPT concentration and RSR, i.e., CPT concentration increased with increasing RSR (relatively more roots). A possible reason is that increased root growth usually is an adaption to increased environmental stress and environmental stress caused more CPT accumulation, so RSR and CPT concentration were positively connected.

Our findings indicated that *C. acuminata* growth and morphology changed significantly two weeks after the N treatments, whereas CPT concentrations did not change until three weeks after the treatments. It is possible that the effect of N treatment on plant growth and morphology occurred prior to its effect on CPT accumulation. In other words, change in CPT accumulation lagged behind the changes in growth and morphology. Similarly, in *C. acuminata* cell culture, CPT accumulation started at the end of the growth phase (Song et al.1998). In a cell suspension

culture of *Berberis parvifolia*, the maximum consumption of nutrients (such as nitrate) took place in the lag phase of the growth cycle, whereas the accumulation of alkaloids occurred in the stationary phase (Deliu et al. 1992). In a similar manner, the alkaloids tabersonine, ajmalicine, and serpentine were distinctly growth associated, with maximum accumulation in the late exponential or stationary growth phase in *C. roseu*s hairy root culture (Bhadra and Shanks 1997).

In conclusion, CPT concentration was not sensitive to external N availability in the growth media in a *C. acuminata* hydroponic culture system. N supplements did not result in an increase in CPT concentration. Conversly, N deficiency resulted in increased CPT concentrations in the leaves and roots. However, CPT yield was the lowest at N 30 mM. CPT concentration was negatively related to plant growth. The effect of N on CPT concentration lagged behind its effect on plant growth and morphology.

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CHAPTER 2. EFFECT OF SALT ON GROWTH, MORPHOLOGY AND CAMPTOTHECIN ACCUMULATION IN *CAMPTOTHECA ACUMINATA* SEEDLINGS

Introduction

Salt as a stress factor has been shown to affect plant growth and plant secondary metabolism in many species. For example, sodium chloride (NaCl) treatment increased the accumulation of ajmalicin alkaloid in *Catharanthus roseus* cell suspension cultures (Carpin et al. 1997). Proline and alkaloid contents in cultured Hyoscyamus cells increased with increasing concentration of NaCl (Saker and El Ashal 1995). We hypothesize that NaCl treatment may also affect CPT accumulation in *Camptotheca acuminata.*

An extensive problem in agriculture is the accumulation of salts from irrigation water. Evaporation and transpiration remove pure water from the soil, and this water loss concentrates solutes in the soil. When irrigation water contains a high concentration of solutes, salts can quickly reach levels that are injurious to salt-sensitive species. *C. acuminata* plantations were established in southern Louisiana, where irrigation caused increased salinity in plantation soils. To establish an effective CPT production system, the examination of the salt effect on plant growth, morphology, and CPT accumulation is of importance.

The objectives of this study were to define the effect of various NaCl levels on plant growth, morphology, CPT concentration, and CPT yield in *C. acuminata,* and to understand the relationships between plant growth, morphology and CPT accumulation.

Materials and Methods

C. acuminata seedlings were propagated in commercial soil plugs from actively expanding shoot tips, with each shoot tip bearing three to four leaves (Liu and Li 2001). These plugs were placed in a hydroponic tray filled with 6 liters of half-strength woody plant medium

(WPM, Lloyd and McCown 1981) solution adjusted to pH 6.5 and supplemented with 2 mg l^{-1} indole butyric acid and enclosed in a mini-chamber. After two to four weeks, the rooted seedlings of similar height (about 10 cm) and similar leaf number (about 6 leaves) were selected and placed into plastic containers containing 100 ml half-strength WPM solution. They were allowed to acclimate in chambers for one week prior to the experiment.

To assess the effect of NaCl on plant growth, morphology and CPT accumulation, thirty seedlings were chosen and divided equally into three groups, and each group was assigned into one of three chambers. In each chamber, seedlings were assigned randomly to a NaCl level of 0, 25, 50, 75, or 100 mM by dispensing NaCl stock solution into seedling container. Each NaCl treatment was replicated twice within each chamber. Seedlings were grown under fluorescent lights at 40 µmol $m^{-2}s^{-1}$ with a 16h photoperiod and at the room temperature (22-26 ºC).

Plant height, weight, leaf number, and blade length of the third leaf from the apex (leaves longer than 1.5 cm were considered to be the first leaves) were used as indicators of plant growth and were measured at the beginning (week 0) and weeks 1, 2, and 3 of the NaCl treatments. Immediately following these measurements, two circular discs (5mm diameter) were punched from each of the 5^{th} and 6^{th} leaves (in the middle of the blade and beside the main vein) on each seedling, put into a 1.5-ml pre-weighed eppendorf tube, frozen in liquid nitrogen, subsequently freeze-dried, and stored in a refrigerator (2-8 °C) for CPT analysis. A piece of medium-sized root was randomly harvested from each seedling at week 3 of the treatments and processed in the same way as the leaf discs for subsequent CPT analysis. Roots, stems, and leaves of each seedling were harvested and weighed to obtain the root to shoot ratio (RSR) at the end of the three-week experiment. The RSR and specific leaf weight (SLW, leaf weight per unit leaf area) obtained from the punched leaf discs were used as indicators of plant morphology.

The freeze-dried leaf and root samples were ground with a pestle in the eppendorf tubes with 25 µl absolute methanol added first to eliminate static electricity, and 600 µl methanol was added additionally into each tube after the grinding. The sealed tubes with plant samples were placed on a rotator for at least 16h at the room temperature (22-26 ºC) in the dark for extraction of CPT. After the sample solids settled to the bottom of the tube, the supernatant was filtered through a 0.2 µm filter (Whatman Inc. Fairfield, NJ) and transferred into a new 1.5-ml tube. The filtrate solution was then dried by air blowing in a hood and reconstituted with 100 µl methanol to achieve a higher concentration of CPT for analysis. Analysis of CPT was performed with a HPLC (Beckman Instruments, Canton, MA) system consisting of a Model 502 autosampler, a Model 125 pump, and a Model 168 photo-diode-array detector as described previously (Liu et al. 1998). CPT concentrations were expressed as a percentage of dry weight. CPT yields were obtained from the leaf CPT concentrations and the total dry leaf weights [leaves were used in nondestructive harvestings (Lopez-Meyer et al. 1994)] at the end of the experiment.

Repeated measures analyses were performed for the NaCl treatment and time effects on plant height, weight, leaf number, leaf length, SLW, leaf CPT concentration, and root CPT concentration. Analyses of variance were performed for the NaCl effect on RSR and CPT yield using SAS software. Least square means (lsmeans) were obtained and compared using least significant difference by Student's t test. Plant growth, morphological, and chemical data were systematically analyzed with covariate analysis with backward variable selection in order to find the relationships between plant growth, morphology and CPT concentration and to identify which variables were significantly related to CPT accumulation. All tests were regarded as significant at $P \leq 0.05$.

Results

Plant Growth

Salt addition of 0, 25, 50, 75 or 100 mM had a significant interaction with time on plant height of *C. acuminata* (Table 2.1). Plant height decreased significantly with increasing NaCl concentration at weeks 1, 2 and 3 of the treatments. The shortest plants were produced at the highest NaCl concentration (100 mM) (Fig. 2.1). Over time, continuous height growth was observed at the low NaCl levels (0, 25 and 50 mM), whereas no height growth was observed at the high NaCl levels (75 and 100 mM).

Similarly, NaCl addition had a significant interaction with time on weight of *C. acuminata* (Table 2.2). NaCl addition significantly affected weight at week 3 of the treatments, where plant weight decreased with increasing NaCl concentration. Plants had the least weight at the highest NaCl level (100 mM) and the greatest weight in the NaCl control (0 mM) (Fig. 2.2).

There was a significant interaction between NaCl and time on leaf number (Table 2.3). Leaf numbers were similar at week 0 and 1, but leaf number decreased significantly with increasing NaCl concentration two and three weeks after the treatments (Fig. 2.3). Leaf number did not increase in seedlings grown in solution containing 75 to 100 mM NaCl after the first week of treatments, whereas leaf number increased continuously when NaCl levels were lowered to 50 mM or less.

The effect of NaCl on leaf length depended on time (Table 2.4). NaCl addition did not affect leaf length at week 1 of the treatments, but significantly affected leaf length at weeks 2 and 3 (Fig. 2.4). Leaf length was significantly reduced by the addition of 75 or 100 mM salt compared to the control at weeks 2 and 3, but leaf length was not affected by NaCl level below 50 mM.

Table 2.1. Repeated measures analysis of NaCl effect on plant height

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	2	23	14.38	<.0001
NaCl	4	23	15.89	<.0001
Time	3	23	124.26	<.0001
Time*NaCl	12	23	15.43	<.0001

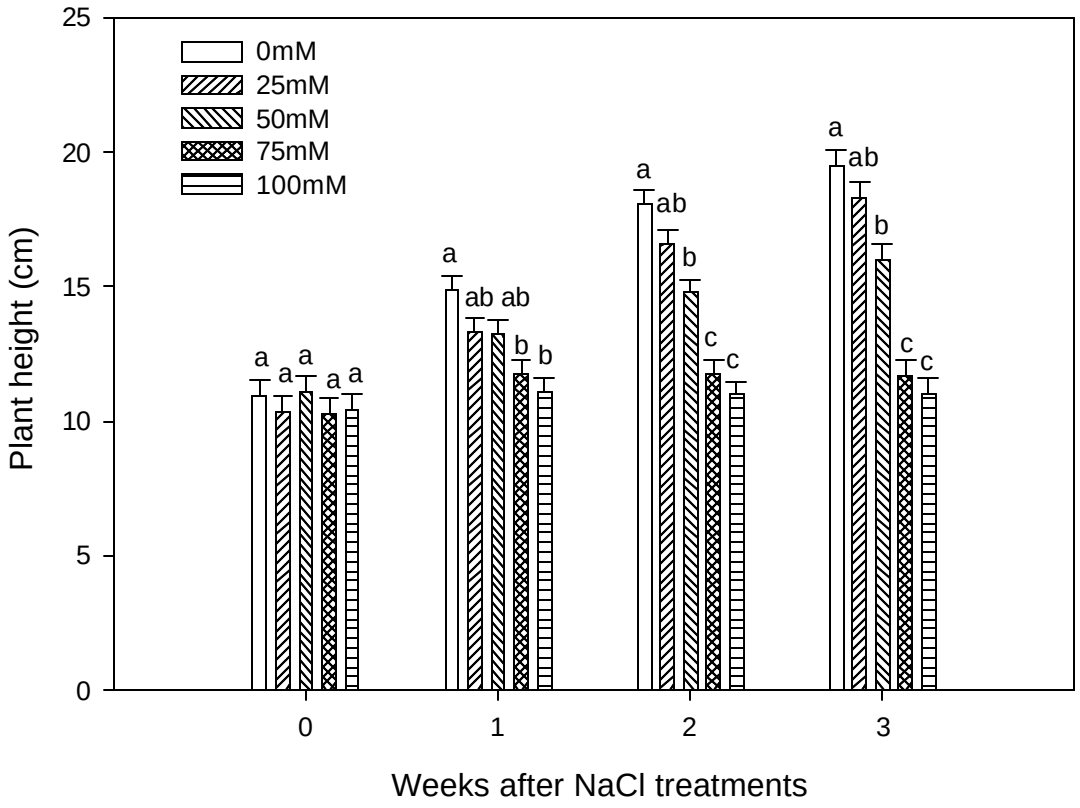

Fig. 2.1. Effect of NaCl on the height of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=6). Different letters above the bars indicate the significant difference of t tests among the lsmeans at $P \leq 0.05$.

Table 2.2. Repeated measures analysis of NaCl effect on plant weight

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	2	23	6.58	0.0055
NaCl	4	23	16.85	<.0001
Time	3	23	156.65	<.0001
Time*NaCl	12	23	27.71	<.0001

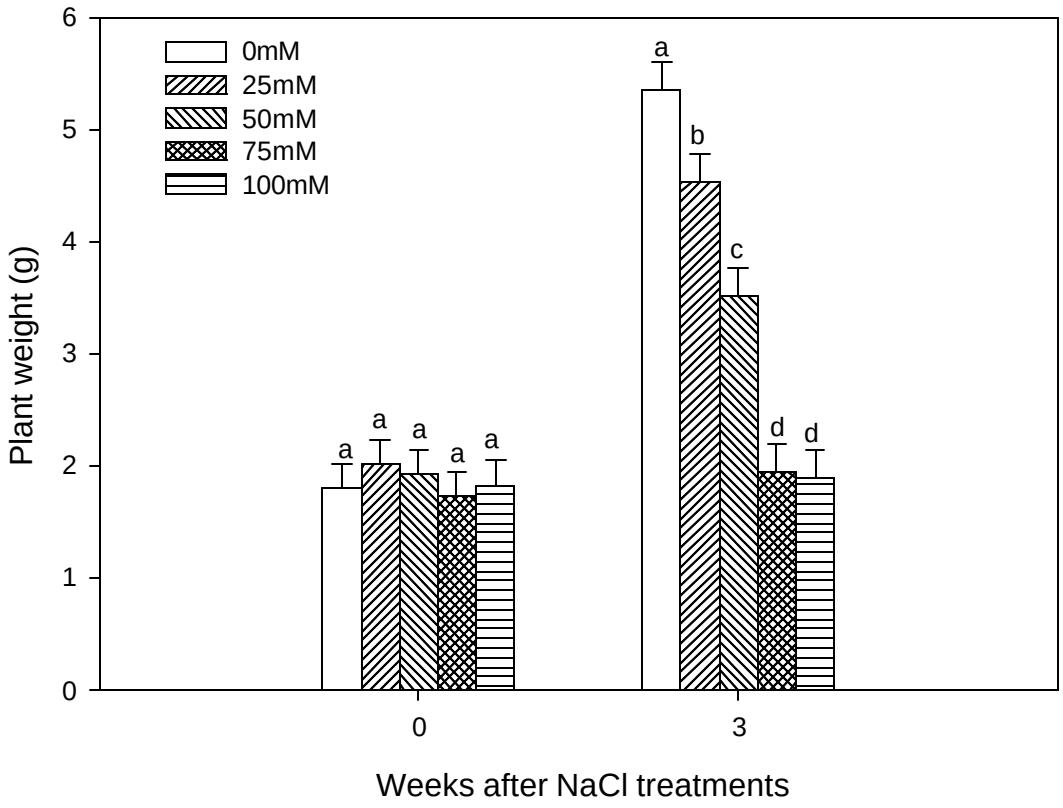

Fig. 2.2. Effect of NaCl on weight of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=6). Different letters above the bars indicate the significant differences of t tests among the lsmeans at $P \leq 0.05$.

Table 2.3. Repeated measures analysis of NaCl effect on leaf number

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	2	23	55.93	<.0001
NaCl	4	23	123.52	<.0001
Time	3	23	3.26	0.0294
Time*NaCl	12	23	4.02	0.0021

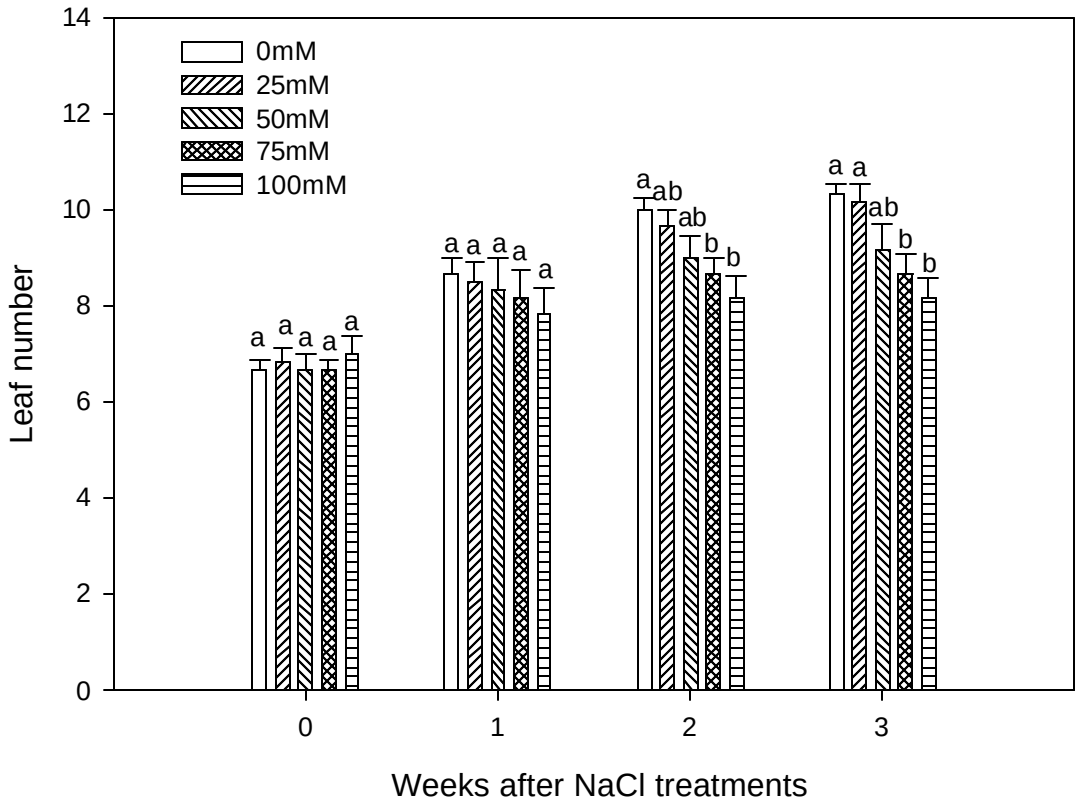

Fig. 2.3. Effect of NaCl on leaf number of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=6). Different letters above the bars indicate the significant differences of t tests among the lsmeans at P ≤0.05.

Table 2.4. Repeated measures analysis of the NaCl effect on leaf length

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	2	23	2.00	0.1582
NaCl	4	23	6.00	0.0018
Time	3	23	72.57	<.0001
Time*NaCl	12	23	8.79	<.0001

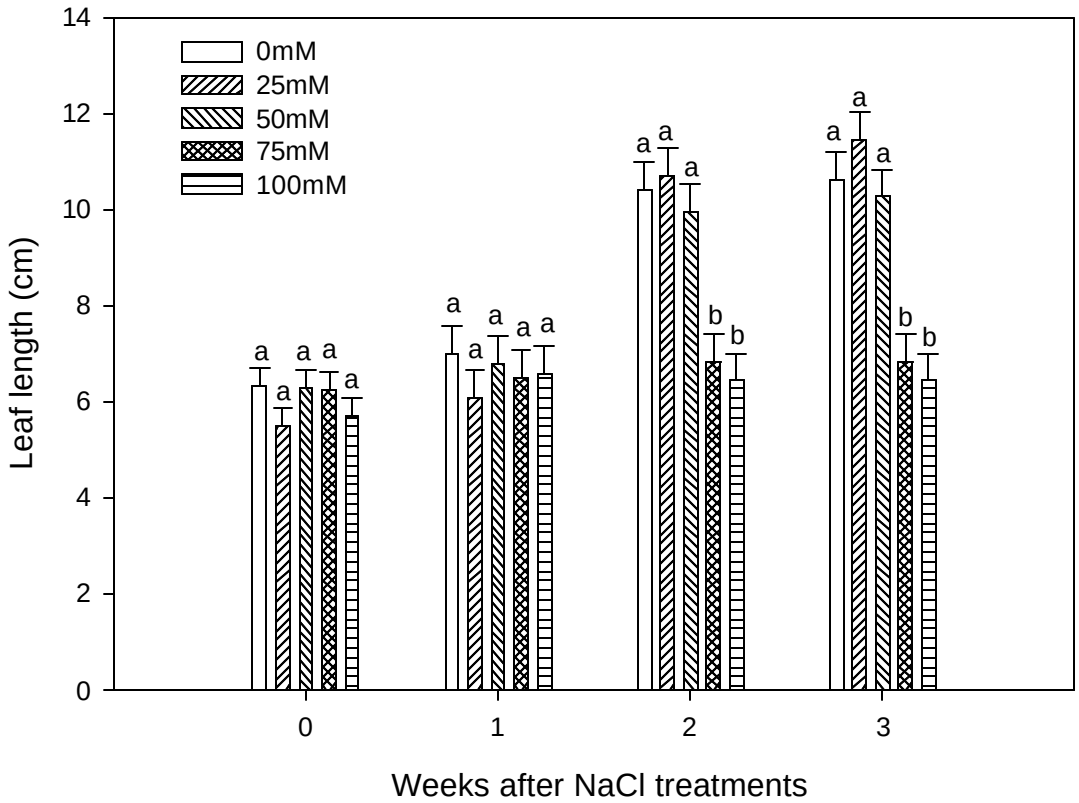

Fig. 2.4. Effect of NaCl on leaf length of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=6). Different letters above the bars indicate the significant differences of t tests among the lsmeans at $P \leq 0.05$.

Plant Morphologic Changes

NaCl addition did not significantly affect the RSR (Table 2.5), although a slight increase in the RSR was observed with increasing NaCl concentration at the end of the experiments (Fig. 2.5).

The effect of NaCl on SLW depended on time (Table 2.6). There were no differences in SLW among all NaCl levels at week 0 of the treatments, but the SLW increased with increasing NaCl concentration at weeks 1 and 3 and higher SLW were produced with NaCl at 75 and 100 mM (Fig. 2.6).

CPT Concentrations in Leaves and Roots and CPT Yield

There was a significant interaction between NaCl addition and time on leaf CPT concentration (Table 2.7). Prior to NaCl treatments, there were no differences in leaf CPT concentrations among all NaCl levels, indicating chemically homogenous experimental materials (Fig. 2.7). There were no observed differences in leaf CPT concentrations among all NaCl levels one and two weeks after the treatments. However, there were significant differences at week 3, where high NaCl levels (75 and 100 mM) produced significantly higher leaf CPT concentrations than low NaCl levels.

NaCl addition significantly affected root CPT concentration (Table 2.8). The highest NaCl level (100 mM) induced significantly higher root CPT concentration than other NaCl levels at week 3 of the treatments (Fig. 2.8).

NaCl addition significantly affected CPT yield at the end of the experiment (Table 2.9). CPT yield decreased with increasing NaCl concentration (Fig. 2.9). The highest CPT yield was produced in the NaCl control.

Table 2.5. Analysis of variance of NaCl effect on RSR

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	2	23	0.35	0.7102
NaCl	4	23	0.84	0.5151

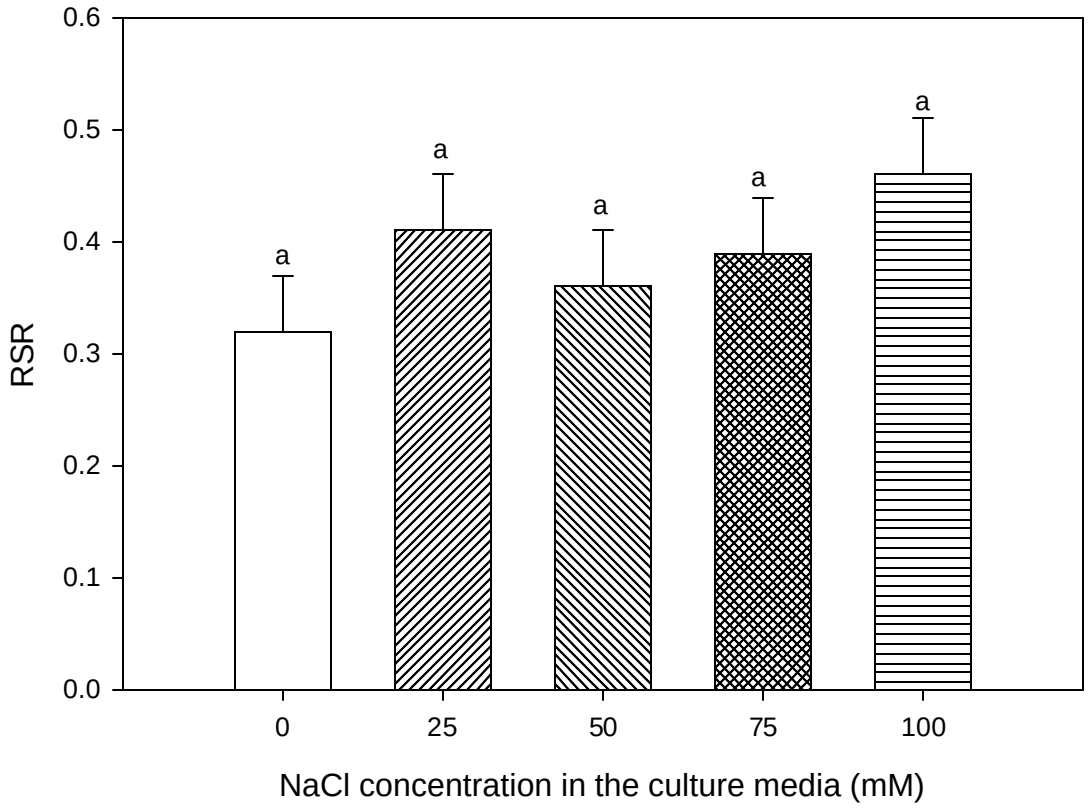

Fig. 2.5. Effect of NaCl on RSR of *C. acuminata* seedlings in a hydroponic culture system at week 3 of the treatments. Vertical lines above each bar represent standard errors of lsmeans (n=6). Different letters above the bars indicate the significant differences of t tests among the lsmeans at $P \leq 0.05$.

Table 2.6. Repeated measures analysis of NaCl effect on SLW

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	2	23	37.57	<.0001
NaCl	4	23	4.73	0.0062
Time	3	23	117.86	<.0001
Time*NaCl	12	23	2.34	0.0386

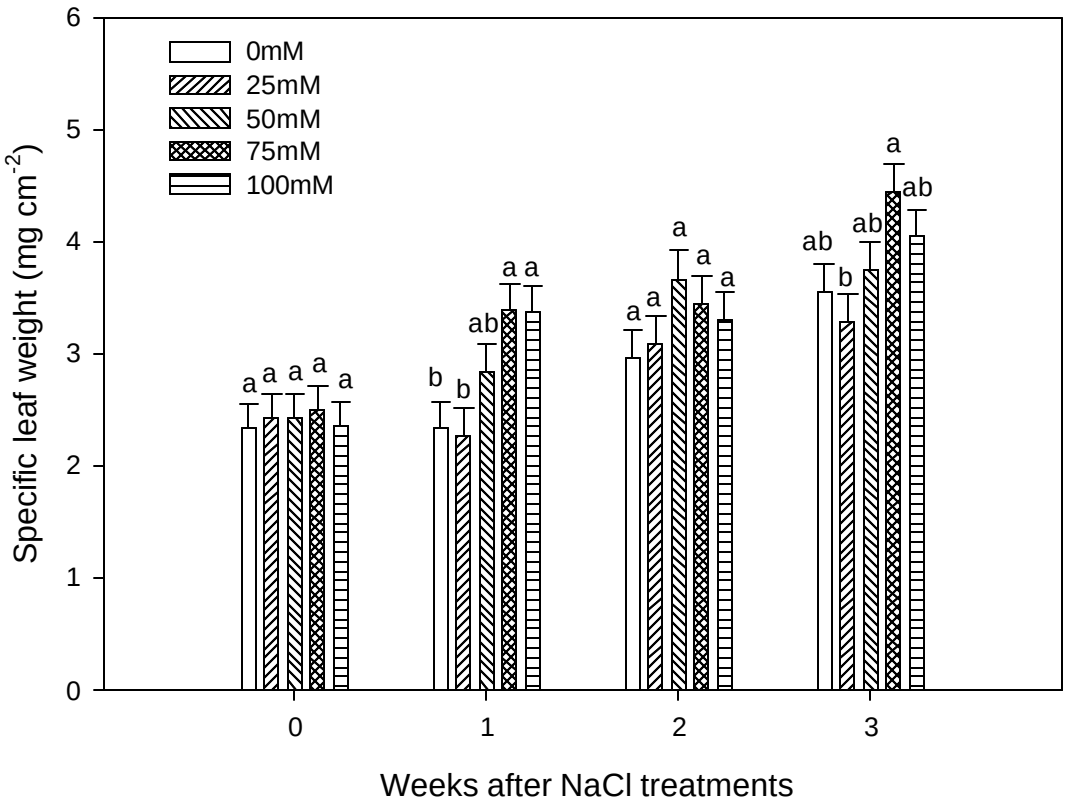

Fig. 2.6. Effect of NaCl on SLW of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=6). Different letters above the bars indicate the significant differences of t tests among the lsmeans at P ≤0.05.

Table 2.7. Repeated measures analysis of NaCl effect on leaf CPT concentration

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	2	23	15.03	<.0001
NaCl	4	23	1.17	0.3508
Time	3	23	7.88	0.0009
Time*NaCl	12	23	3.15	0.0088

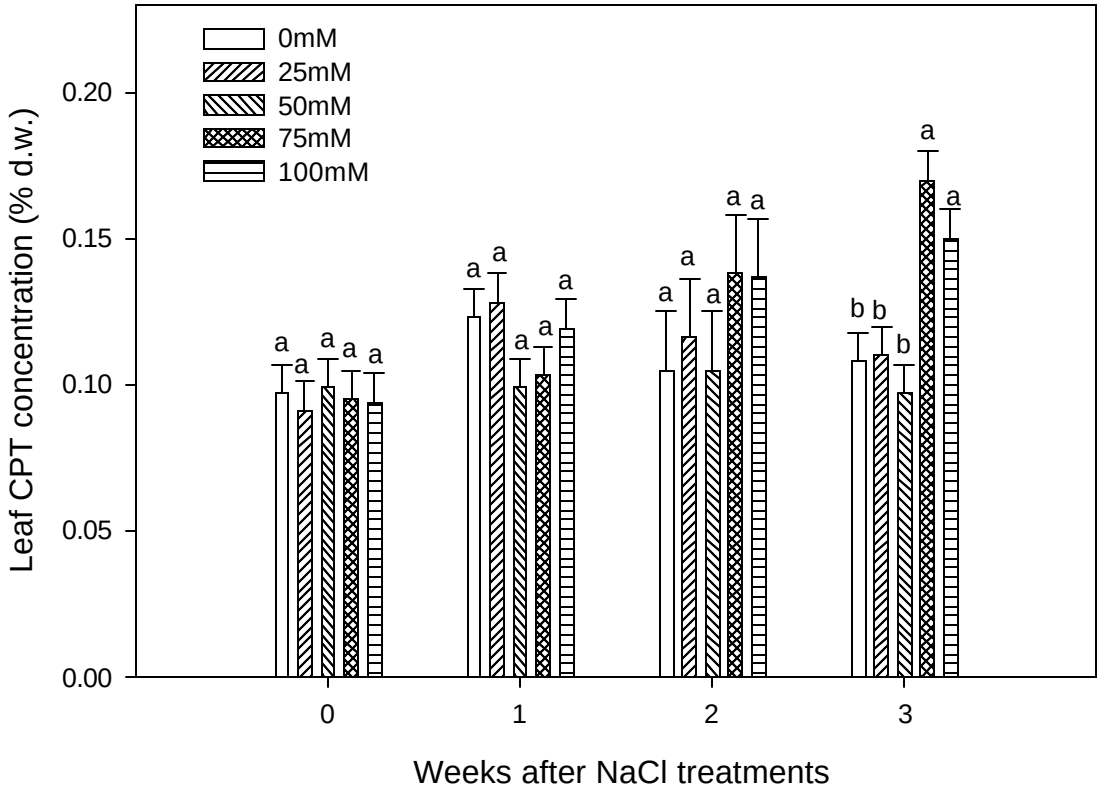

Fig. 2.7. Effect of NaCl on leaf CPT concentration of *C. acuminata* seedlings in a hydroponic system. Vertical lines above each bar represent standard errors of lsmeans (n=6). Different letters above the bars indicate the significant differences of t tests among the lsmeans at P ≤0.05.

Table 2.8. Analysis of variance of NaCl effect on root CPT concentration

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	2	23	0.69	0.5105
NaCl	4	23	3.86	0.0153

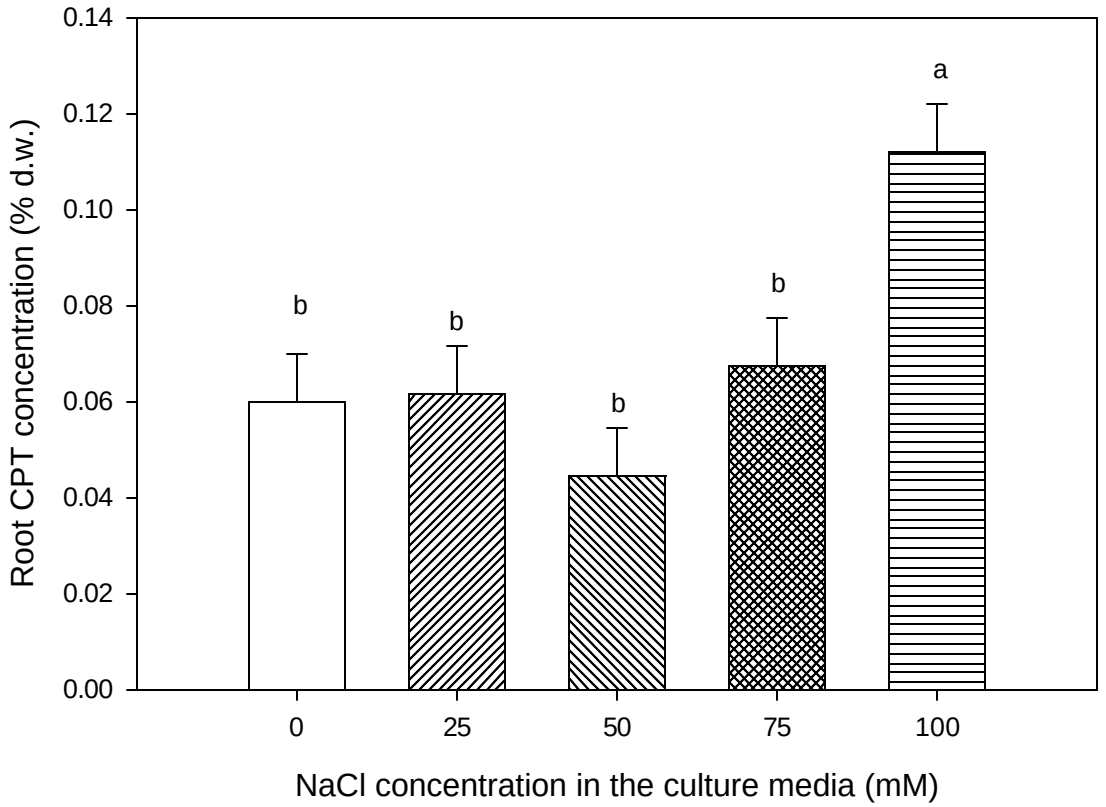

Fig. 2.8. Effect of NaCl on root CPT concentration of *C. acuminata* seedlings in a hydroponic system at week 3 of the treatments.Vertical lines above each bar represent standard errors of lsmeans (n=6). Different letters above the bars indicate the significant differences of t tests among the lsmeans at P ≤0.05.

Table 2.9. Analysis of variance of NaCl effect on CPT yield

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	2	23	3.94	0.0339
NaCl	4	23	7.87	0.0004

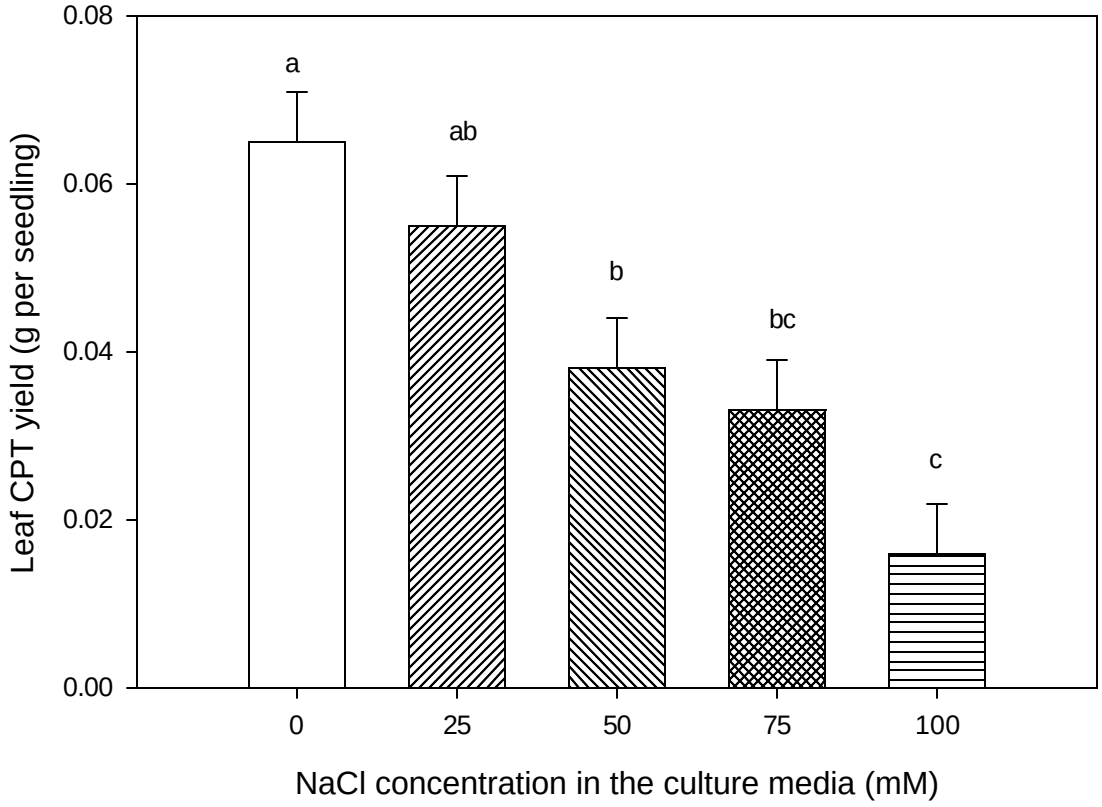

Fig. 2.9. Effect of NaCl on CPT yield of *C. acumianta* seedlings in a hydroponic culture system at week 3 of the treatments. Vertical lines above each bar represent standard errors of lsmeans (n=6). Different letters above the bars indicate the significant difference of t tests among the lsmeans at P ≤0.05.

CPT Concentration and Plant Growth, Morphology

Covariate analysis with backward variable selection among plant height, weight, leaf number, leaf length, RSR and SLW showed that CPT concentration had a significant relation with the leaf number (Table 2.10). A negative relationship between CPT concentration and leaf number was found.

Table 2.10. Covariate analysis with backward variable selection for NaCl study

Variable	Estimate	Type II SS	F-value	P-value
Intercept	0.26581	0.04318	17.23	0.0003
Leaf number	-0.01451	0.01111	4.43	0.0443

Discussion and Conclusions

Plant Growth

NaCl addition significantly reduced plant height, weight, leaf number, and leaf length of *C. acuminata.* This suggests that salt stress has a detrimental effect on plant growth. A possible reason might be that salt addition increased the accumulation of sodium ions (Na^+) and/or chloride ions (Cl^-) in plant tissues and/or organs, and produced osmotic stress to inhibit plant growth, as in other plant species (Ali 2000, Saker and EI Ashal 1995). The continuous increase in height over time with NaCl concentrations below 75 mM, but no increase in height over time with NaCl concentrations above 75 mM may suggest that a concentration of 75 mM is perhaps a threshold for NaCl to exert negative effects on *C. acuminata* seedling growth. Seedlings under the threshold of 75 mM NaCl could continue to grow over the three weeks, presumably through their physiological adjustment. It was thought that tolerance to salt stress in plants is through a common mechanism of Na^+ uptake for osmotic adjustment (Glenn and Brown 1998). Short-term osmotic adjustment enables plants to grow under high salinity conditions (Thomas and Bohnert 1993).

Plant Morphologic Changes

NaCl addition led to a trend of increased RSR and higher SLW in our study. Similarly, a rapid increase in roots was also observed in other plant species under salt stress (Koyro 2000). The changes in plant morphology might be results of adaptations to environmental stress. The increased RSR, or more roots, might be an adaptation by the plant to the osmotic stress because more roots could be useful to promote water uptake. The increased SLW or more fibrous leaves might create a physical barrier to further water loss.

CPT Concentration and Possible Mechanism

Below the threshold of 75 mM NaCl, plant growth was reduced with increasing NaCl levels, but CPT concentrations did not change. This suggested that plant secondary metabolite accumulation remained unaffected by NaCl levels below 75 mM despite the fact that growth was adversely affected. One possible reason might be that at low NaCl levels, the plant did not accumulate Na^+ and/or Cl^- ions in their organs or vacuoles to the toxic levels necessary to activate the plant defense system for CPT biosynthesis.

Above the threshold of 75 mM, there was no measurable plant growth one and two weeks after the NaCl treatments. The CPT concentrations were unchanged at weeks 1 and 2 in comparison to the NaCl control, but increased significantly at week 3. A possible explanation might be that Na^+ and/or Cl^- ions accumulated in plant tissues and/or organs produced osmotic stress, but the Na^+ and/or Cl^- ion concentrations were not high enough to induce the CPT defense system at weeks 1 and 2. The continuous accumulation of Na^+ and/or Cl^- ions in tissues and/or organs following the first and second week might have induced the plant defense system to produce more CPT. It was possible that an ion accumulation mechanism might be used for inducing the plant defense system and CPT accumulation. Similarly, exposure of cells to an

osmotic stress caused by Na^+ and Cl^- ions accumulation produced a marked increase (320%) in the total alkaloid content (Godoy-Hernandez and Loyola-Vargas 1994). Endogenous methyl jasmonate (MeJa) was implicated as a regulator of the expression of salt stress-inducible proteins, and MeJa levels showed a differential increase with the dose and the duration of salt stress. The endogenous MeJa accumulations only exhibited a marked increase when plants were exposed to high NaCl concentrations (>150 mM), but remained unchanged when plants were exposed to NaCl below 125 mM (Moons et al. 1997).

After accumulation of Na^+ and/or Cl^- ions, certain plant defense genes might be activated by internal osmotic stress. NaCl stress increased the accumulation of transcripts from a gene of cytosolic cyclophiline, an enzyme responsible for alkaloid production in *Catharanthus rosues* (Droual et al. 1997). Likewise, a salt tolerant line of *C. roseus* produced more alkaloid, which suggested that the gene responsible for stress response might have been activated, resulting in increased alkaloid biosynthesis (Vazquez and Loyola 1994). In our study, both leaf and root CPT concentrations increased in response to osmotic stress at high NaCl concentrations. We hypothesize that the CPT chemical defense system was activated under the osmotic stress. As a secondary metabolite, CPT serves mainly as a defensive chemical in response to abiotic and biotic stress in *C. acuminata* (Liu et al. 1998). Two programmed chemical defense systems have been found in *C. acuminata* (Lopez-Meyer and Nessler 1997, Maldonado-Mendoza et al.1997). One CPT defense system is employed during ontogenic development with corresponding genes *tdc1* and *hmg2, hmg3*. Another CPT defense system is employed in response to abiotic and biotic stresses with corresponding genes *tdc2* and *hmg1*. Salt stress might have activated the corresponding *tdc2* and *hmg1* genes.

Our finding that NaCl at high concentrations increased CPT concentrations both in leaves and roots after a period of three weeks may suggest that NaCl can be used as a stress factor for inducing the accumulation of the defense chemicals, such as CPT.

CPT Yield

Final leaf CPT yield decreased with increasing NaCl concentrations and the highest CPT yield was in the NaCl control. Final CPT yield in leaves depended on two factors: plant biomass and CPT concentration. Although high NaCl levels produced higher CPT concentration, they had much lower plant biomass and so it produced lower CPT yield. On the other hand, the NaCl control produced the highest CPT yield, because it had similar CPT concentration as low NaCl levels but it had the highest biomass.

CPT Concentration and Plant Growth, Morphology

In this salt study, covariate analysis indicated that there was a significant relation between CPT concentration and leaf number. The negative relation between CPT concentration and leaf number might suggest that CPT accumulation increased with decreasing plant growth, e.g., leaf number under salt stress. A possible explanation may be that plant defense to stress may require reallocation of total resources; therefore, an alternative supply or balance between growth and defense according to environments probably exists within plants (van der Meijden et al. 1988).

In conclusion, NaCl addition significantly reduced plant height, weight, leaf number and leaf length of *C. acuminata* seedlings in a hydroponic culture system, but increased SLW. Only high NaCl concentrations caused elevated CPT concentrations both in leaves and roots. However, the CPT yields decreased with increasing NaCl levels. CPT concentration had a negative relation with plant growth under the salt stress.

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CHAPTER 3. EFFECT OF UV LIGHT ON GROWTH, MORPHOLOGY, AND CAMPTOTHECIN ACCUMULATION IN *CAMPTOTHECA ACUMINATA* SEEDLINGS

Introduction

It is well known that as a stress factor UV light inhibits plant growth, affects plant morphology, and further affects plant secondary metabolism during lengthy exposure. For example, artificially increased levels of UV radiation caused significantly shorter but a greater number of leaves, and UV light significantly increased the accumulation of flavonoids, a class of secondary metabolite in *aquilegia spp.* leaves (Larson et al. 1990). UV light also stimulates the biosynthesis of alkaloids, another class of secondary metabolite as demonstrated in the following two examples. Artificial near-ultraviolet light at a peak of 370 nm with 1200 $\mu w\ cm^{-2}$ stimulated the biosynthesis of dimeric indole alkaloids in intact seedlings of *Catharanthus roseus* through the stimulation of an FMN-mediated, non-enzymatic coupling of vindoline and catharanthine to form an intermediate, the precursor of the dimeric alkaloid synthesis (Hirata et al. 1993). Under irradiation with near-ultraviolet light at a peak of 370 nm, the levels of leurosine and vinblastine in multiple shoot culture of *C. roseus* also increased (Hirata et al. 1992).

CPT biosynthesis needs nitrogen (N) salt as its ultimate N source, and as a stress factor, UV light may stimulate CPT biosynthesis, which may utilize internal and external N resources. It may be conceivable that if more N resources (N salt) is supplied in the media and absorbed by *C. acuminata* plants and UV light is applied to the plants, UV stress may stimulate CPT biosynthesis and N salt may provide more resources for CPT biosynthesis without N becoming a limiting factor. In other words, UV light and N salt may have synergistic interaction on CPT biosynthesis.

We hypothesize that when N is enriched in growth media and *C. acuminata* plants are exposed to UV light, more CPT will accumulate in the plants. The objectives of this study were to examine the effect of UV light on plant growth, morphology, CPT concentration, and CPT yield in *C. acuminata* seedlings, to find the interaction between UV light and N salt, and to identify the relationships between plant growth, morphology, and CPT accumulation.

Materials and Methods

C. acuminata seedlings were propagated in commercial soil plugs from actively expanding shoot tips bearing three to four leaves (Liu and Li 2001). These plugs were placed in a hydroponic tray filled with 6 liters of half-strength woody plant medium (WPM, Lloyd and McCown 1981) solution adjusted to pH 6.5, supplemented with 2 mg l^{-1} indole butyric acid, and enclosed in a mini-chamber. After two to four weeks, the rooted seedlings with similar height (about 9 cm) and similar leaf number (about 7 leaves) were selected and placed into plastic containers containing 100 ml of half-strength WPM solution. Seedlings were allowed to acclimate for one week in the chambers prior to the experiment.

To assess UV light and UV-N interaction on plant growth, morphology, and CPT accumulation, thirty-six seedlings were chosen and divided into three groups. A split-plot design was used with UV as the main plot and N as the split-plot. In each group, seedlings were divided equally and put into 2 chambers, one of which received dark treatment and another received UV light. In each chamber, seedlings were assigned randomly to one of two N treatments (7.5 or 15 mM). The experiment was conducted as follows: Half-strength WPM solution with a volume of 3.6 liters was prepared and divided equally into two flasks. The N salts made by NH_4NO_3: $Ca(NO_3)_2$ with a molar ratio of 2:1 were then added to one flask to double the N concentration to 15 mM. A 100 ml aliquot from each of the two flasks was dispensed into a seedling container.

The N treatments were replicated three times within each of 6 chambers, and all chambers were put underneath fluorescent lights at 40 μmol $m^{-1}s^{-1}$ intensity with a 16h photoperiod. Three of the 6 chambers were selected to receive 2h of UV light at a peak of 254 nm and light intensity of 1100 μw cm^{-2} at a distance of 25 cm every night, whereas the other three chambers received dark treatment.

Plant height, weight, leaf number, and blade length of the third leaf from the apex (leaves longer than 1.5 cm were considered as the first leaves) were used as indicators of plant growth and were measured at the beginning and at weeks 1, 2, and 3. Two circular discs (5mm diameter) were punched from each of the 5^{th} and 6^{th} leaves (in the middle of the blade and beside the main vein) on each seedling, put into a 1.5-ml pre-weighed eppendorf tube, frozen in liquid nitrogen, subsequently freeze-dried, and stored in a refrigerator (2-8 °C) for CPT analysis. The roots, stems, and leaves of each seedling were harvested and weighed to obtain the root to shoot ratio (RSR) at the end of the 3-week experiment. The RSR and specific leaf weight (SLW, leaf weight per unit leaf area) obtained from the punched leaf discs were used as indicators of plant morphology.

The freeze-dried leaf samples were ground with a pestle in eppendorf tubes with 25 μl absolute methanol added first to eliminate static electricity, and 800 μl methanol was added after the grinding. The samples in locked tubes were extracted for CPT on a rotator for at least 16h at room temperature (22-26°C) in dark. After sample solids settled to the bottom of the tube, the supernatant was filtered through a 0.2 μm filter (Whatman Inc. Fairfield, NJ) and transferred into a new 1.5-ml tube. The filtrate solution was dried by air blowing under a hood and reconstituted with 100 μl methanol to achieve a higher concentration of CPT for chemical analysis. Analysis of CPT was performed with a HPLC system (Beckman Instruments, Canton, MA) consisting of a

Model 502 autosampler, a Model 125 pump, and a Model 168 photo-diode-array detector as previously described (Liu et al. 1998). CPT concentrations were expressed as a percentage of dry weight. CPT yields were obtained from the leaf CPT concentrations and the total dry leaf weights [leaves were used in nondestructive harvestings (Lopez-Meyer et al. 1994)] at the end of the experiment.

Repeated measures analyses were performed for the UV-N and time effects on plant height, weight, leaf number, leaf length, SLW, and leaf CPT concentration. Analyses of variance were performed for the UV-N effect on RSR and CPT yield using SAS software. Least square means (lsmeans) were obtained and compared using least significant difference by Student's t test. Plant growth, morphological, and chemical data were systematically analyzed with covariate analysis with backward variable selection in order to find the relationships between plant growth, morphology and CPT concentration and to identify which variables were significantly related to CPT accumulation. All tests were regarded as significant at $P \leq 0.05$.

Results

Plant Growth

There was no significant interaction between UV light and N treatments on height of *C. acuminata* and there was also no significant interaction between UV light and time on height of *C. acuminata* (Table 3.1). UV-treated seedlings had similar heights as UV-untreated seedlings throughout the 3-week experiment (Fig. 3.1).

There was no significant interaction between UV light and N treatments on weight of *C. acuminata* and there was also no significant interaction between UV light and time on weight of *C. acuminata* (Table 3.2). Plant weights were similar between UV-treated and UV-untreated seedlings one, two, and three weeks after the treatments (Fig. 3. 2).

Table 3.1. Repeated measures analysis of UV light effect on plant height

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Group	2	30	5.37	0.0101
UV	1	30	2.79	0.1052
N	1	30	0.62	0.4380
UV*N	1	30	3.28	0.0801
Time	3	30	523.08	<.0001
Time*UV	3	30	0.46	0.7136

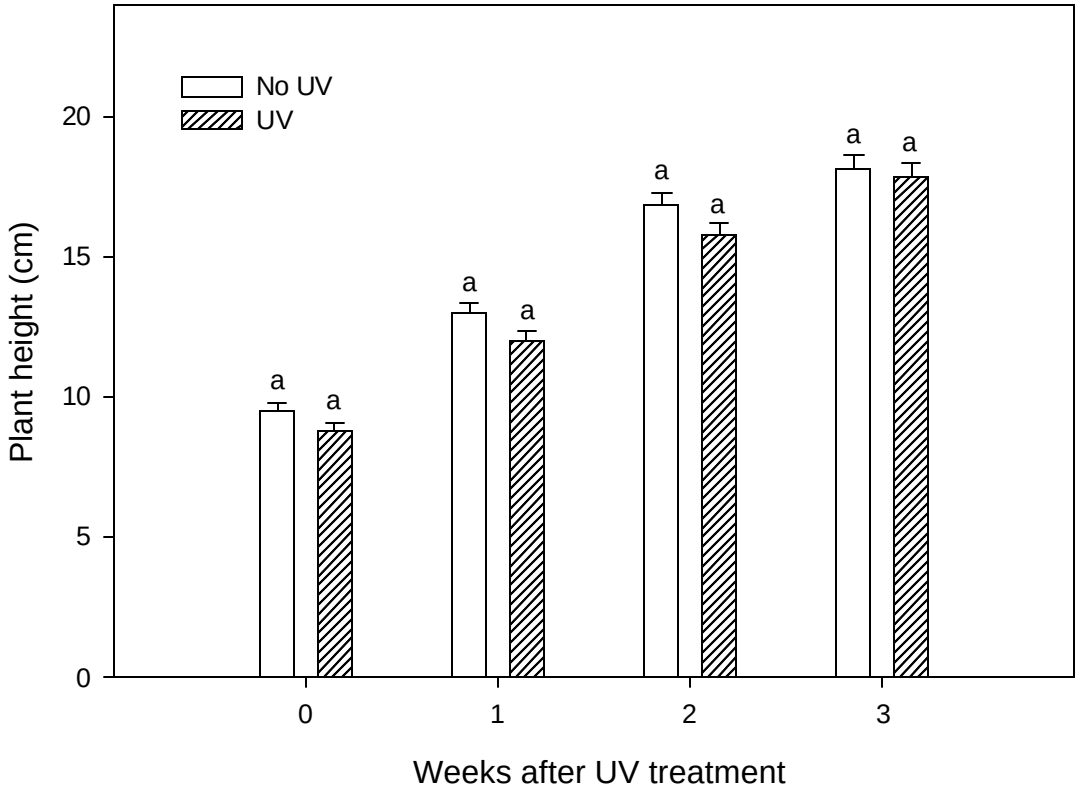

Fig. 3.1. Effect of UV light on height of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=9). Different letters above the bars indicate the significant difference of the t test between the lsmeans at P ≤0.05.

Table 3.2. Repeated measures analysis of UV effect on plant weight

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Group	2	30	3.27	0.0519
UV	1	30	0.03	0.8604
N	1	30	0.16	0.6895
UV*N	1	30	0.04	0.8473
Time	3	30	151.83	<.0001
Time*UV	3	30	0.52	0.6685

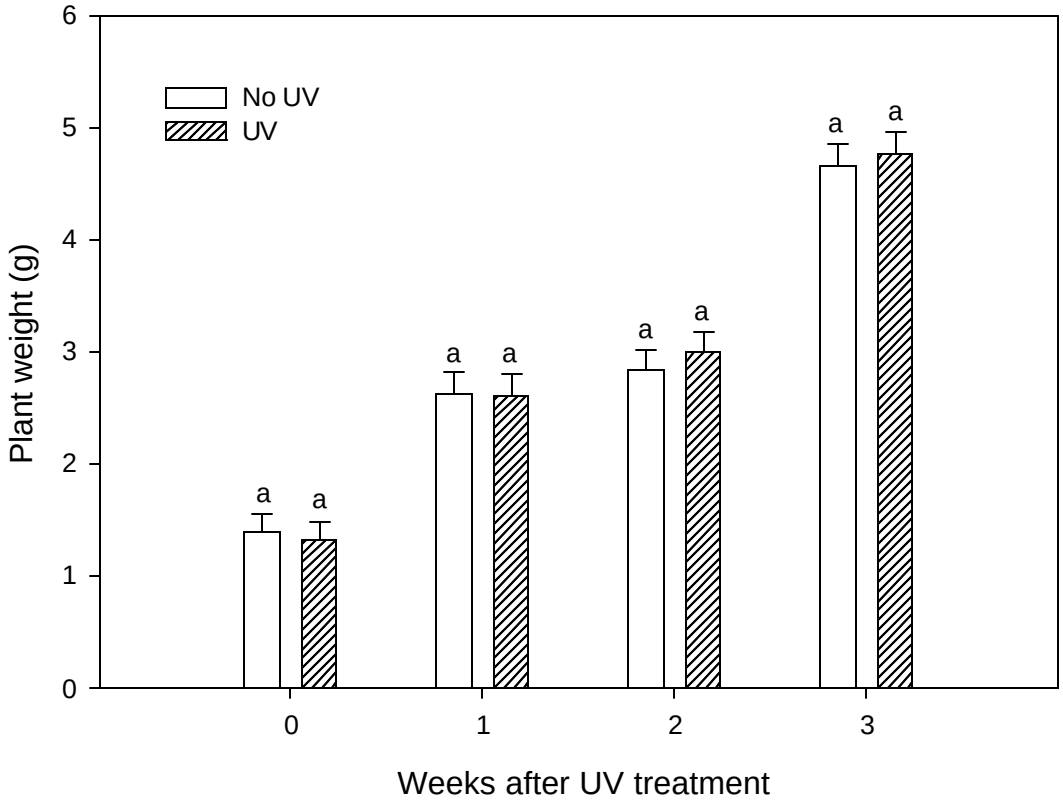

Fig. 3.2. Effect of UV light on weight of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=9). Different letters above the bars indicate the significant difference of t tests among the lsmeans at $P \leq 0.05$.

UV light had no significant interaction with N treatments on leaf number and UV light also had no significant interaction with time on leaf number (Table 3.3). UV had no effect on leaf number through out the three weeks (Fig. 3.3).

UV light had no significant interaction with N treatments on leaf length and UV light had also no significant interaction with time on leaf length (Table 3.4). At the same time, UV light exposure did not affect leaf length compared to the control throughout the three weeks of the experiment (Fig. 3.4).

Morphological Changes

UV light had no significant interaction with N treatments on RSR of *C. acuminata* (Table 3.5). UV treatment did not affect the RSR three weeks after the treatments (Fig. 3.5).

There was no significant interaction between UV light and N on SLW and there was also no significant interaction between UV light and time on SLW (Table 3. 6). The SLW did not change at weeks 1, 2, and 3 of the treatments, whereas a slight decrease in SLW was observed in seedlings with the UV light exposure (Fig. 3.6).

CPT Concentrations in Leaves and CPT Yield

There was no significant interaction between UV light and N treatments on leaf CPT concentration, but there was a significant interaction between UV light and time on leaf CPT concentration (Table 3.7). Prior to UV treatment, there were no differences in CPT concentrations among the treatments (Fig. 3.7). UV-treated seedlings had similar CPT concentrations as UV-untreated ones at weeks 1 and 2 of the treatments. However, UV-treated seedlings had significantly higher CPT concentrations than UV-untreated seedlings at week 3 of the treatments.

Table 3.3. Repeated measures analysis of UV light effect on leaf number

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Group	2	30	5.50	0.0092
UV	1	30	0.26	0.6148
N	1	30	0.10	0.7521
UV*N	1	30	0.05	0.8169
Time	3	30	421.79	<.0001
Time*UV	3	30	0.31	0.8175

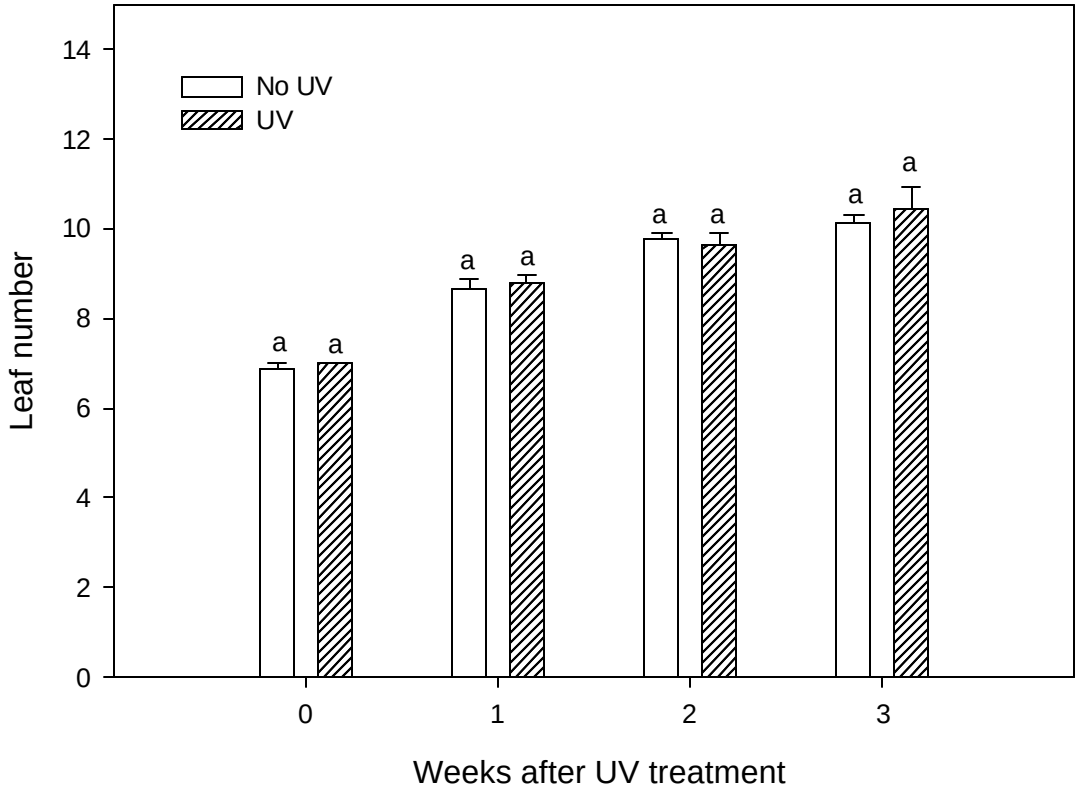

Fig. 3.3. Effect of UV light on leaf number of *C. acuminata* seedlings a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=9). Different letters above the bars indicate the significant differences of t tests among the lsmeans at P ≤0.05.

Table 3. 4. Repeated measures analysis of UV effect on leaf length

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Group	2	30	16.62	<.0001
UV	1	30	0.08	0.7825
N	1	30	3.49	0.0714
UV*N	1	30	2.87	0.1006
Time	3	30	170.62	<.0001
Time*UV	3	30	2.37	0.0905

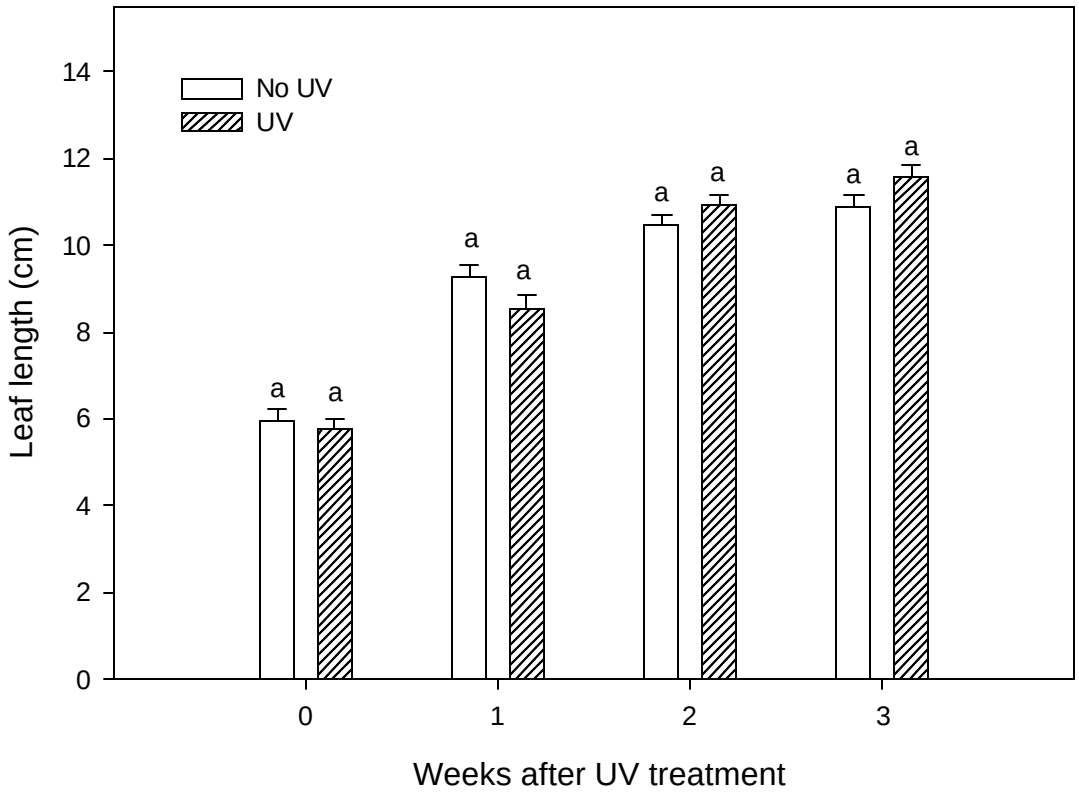

Fig. 3.4. Effect of UV light on leaf length of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=9). Different letters above the bars indicate the significant differences of t tests among the lsmeans at P ≤0.05.

Table 3.5. Analysis of variance of UV light effect on RSR

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Group	2	30	0.48	0.6228
UV	1	30	0.00	0.9773
N	1	30	4.96	0.0336
UV*N	1	30	0.20	0.6559

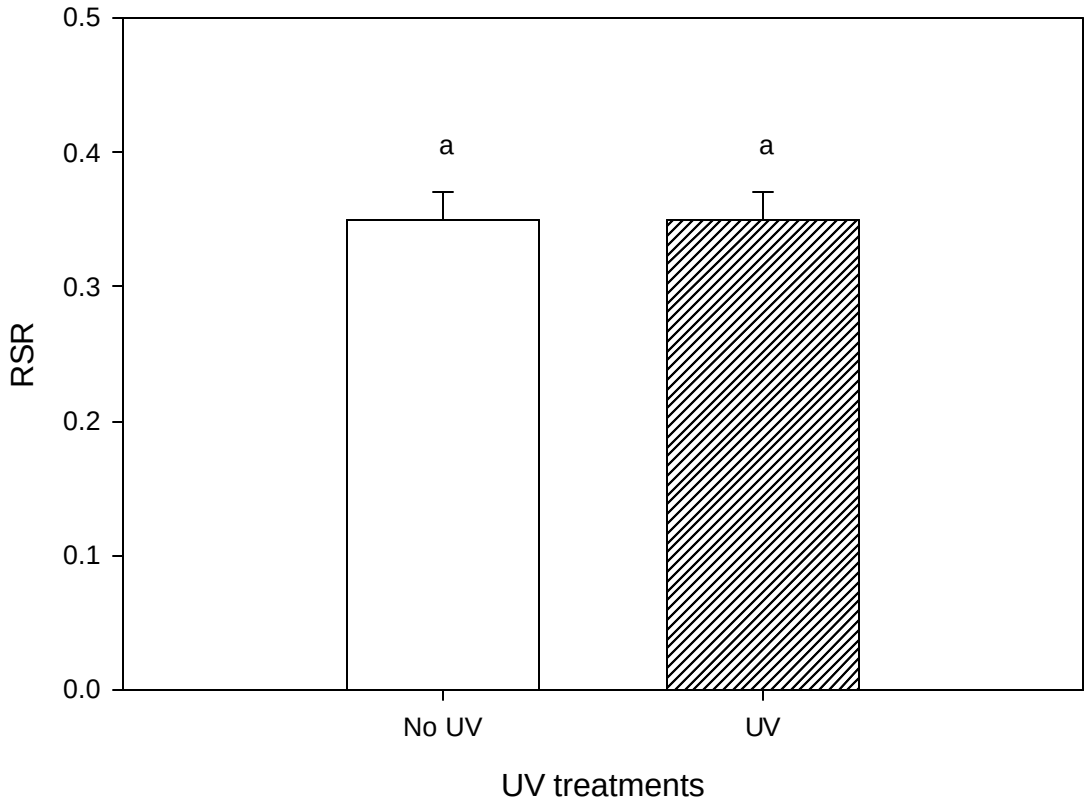

Fig. 3.5. Effect of UV light on RSR of *C. acuminata* seedlings in a hydroponic culture system at week 3 of the treatments. Vertical lines above each bar represent standard errors of lsmeans (n=9). Different letters above the bars indicate the significant difference of t tests among the lsmeans at P≤0.05.

Table 3.6 Repeated measures analysis of UV light effect on SLW

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Group	2	30	0.21	0.8140
UV	1	30	1.57	0.2195
N	1	30	1.19	0.2849
UV*N	1	30	3.10	0.0884
Time	3	30	5.54	0.0090
Time*UV	3	30	2.18	0.1308

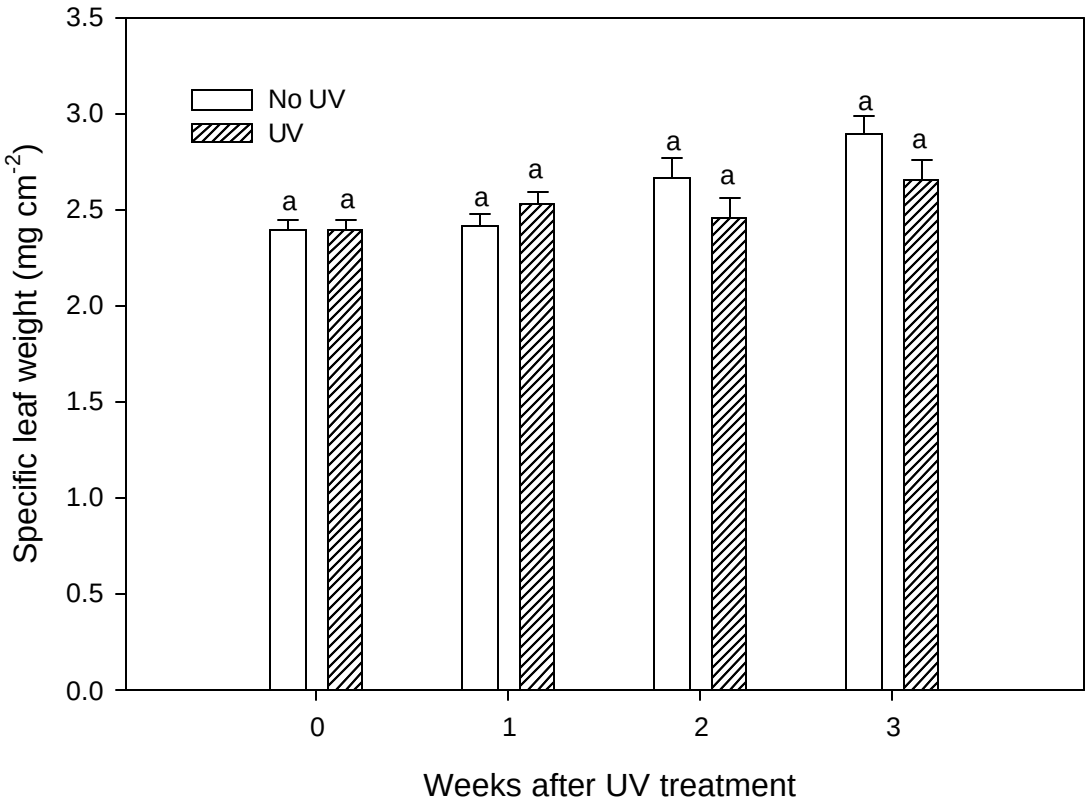

Fig. 3.6. Effect of UV light on SLW of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=9). Different letters above the bars indicate the significant difference of t tests among the lsmeans at P≤0.05.

Table 3.7. Repeated measures analysis of UV light effect on leaf CPT concentration

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Group	2	30	5.92	0.0068
UV	1	30	2.62	0.1163
N	1	30	1.21	0.2810
UV*N	1	30	1.95	0.1732
Time	3	30	2.80	0.0684
Time*UV	3	30	2.92	0.0504

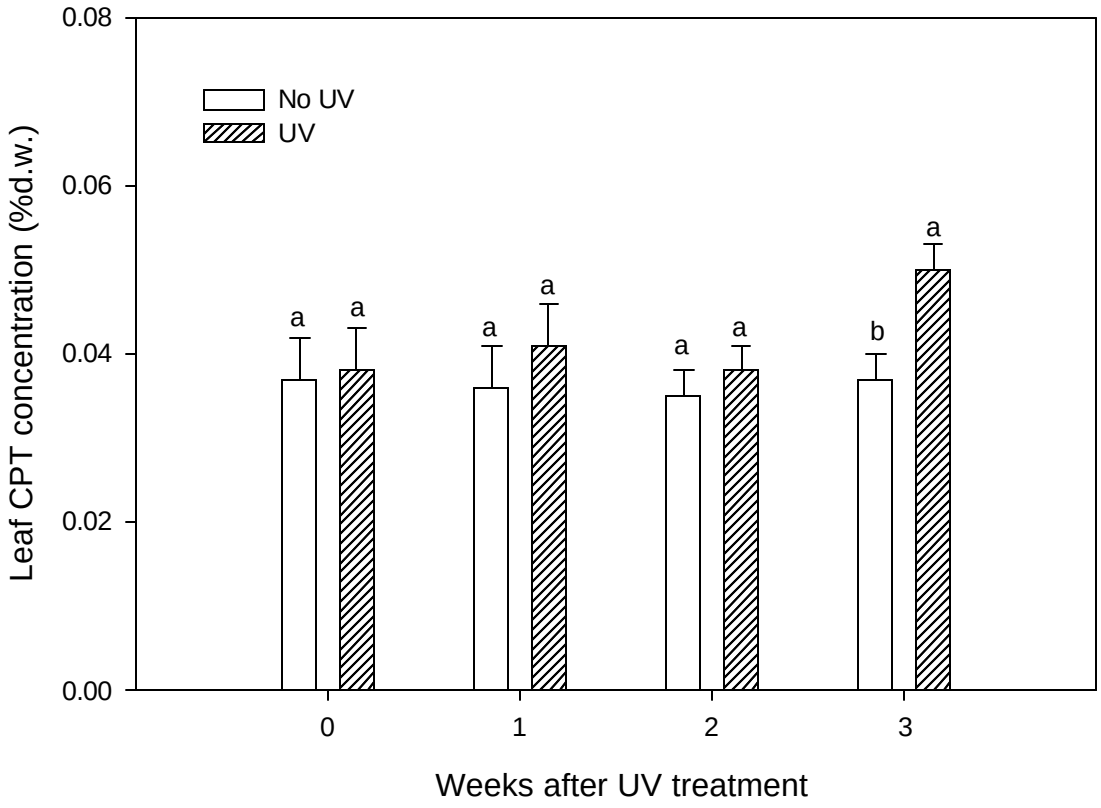

Fig. 3.7. Effect of UV light on leaf CPT concentration of *C. acuminata* seedlings in a hydroponic system. Vertical lines above each bar represent standard errors of the lsmeans (n=9). Different letters above the bar indicate significant difference of t tests among lsmeans at P≤0.05.

There was no significant interaction between UV light and N treatments on CPT yield, but there was a significant effect of UV light on CPT yield (Table 3.8). UV treated seedling had significantly higher CPT yield than UV untreated seedlings (Fig. 3.8).

CPT Concentration and Plant Growth, Morphology

Covariate analysis with backward variable selection among plant height, weight, leaf number, leaf length, RSR, and SLW was done (Table 3.9). RSR and SLW were slightly related to CPT concentration. A slight positive relation was found between CPT concentration and RSR, and a slight negative relation was found between CPT concentration and SLW.

Discussion and Conclusions

Plant Growth

UV light in this study had no effect on plant growth in height, weight, leaf number, and leaf length. A possible reason might be that in a short-term exposure (2h every day) and in a short period (3 weeks) UV light was not strong enough to inhibit plant growth, although leaf distortion and discoloration were observed in our experiment. Similarly, in the *in vitro* culture of carrot cells, the measured growth parameters did not show any changes under UV exposure (Broetto and Crocomo 1995).

Plant Morphologic Change

The results of this experiment indicated that UV light had no effect on RSR, and UV treated seedlings had slightly less SLW in leaves than UV untreated seedlings. It might suggest that UV light exposure did not affect the seedlings' root and shoot growth or the allocation of photosynthates within seedlings. The less SLW or lighter leaves might be a result of UV light's detrimental effect on leaf tissues, since leaf distortion and discoloration in response to the UV light exposure had been observed.

Table 3.8. Analysis of variance of UV light effect on CPT yield

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Group	2	30	2.84	0.0740
UV	1	30	4.75	0.0373
N	1	30	0.04	0.8455
UV*N	1	30	0.00	0.9498

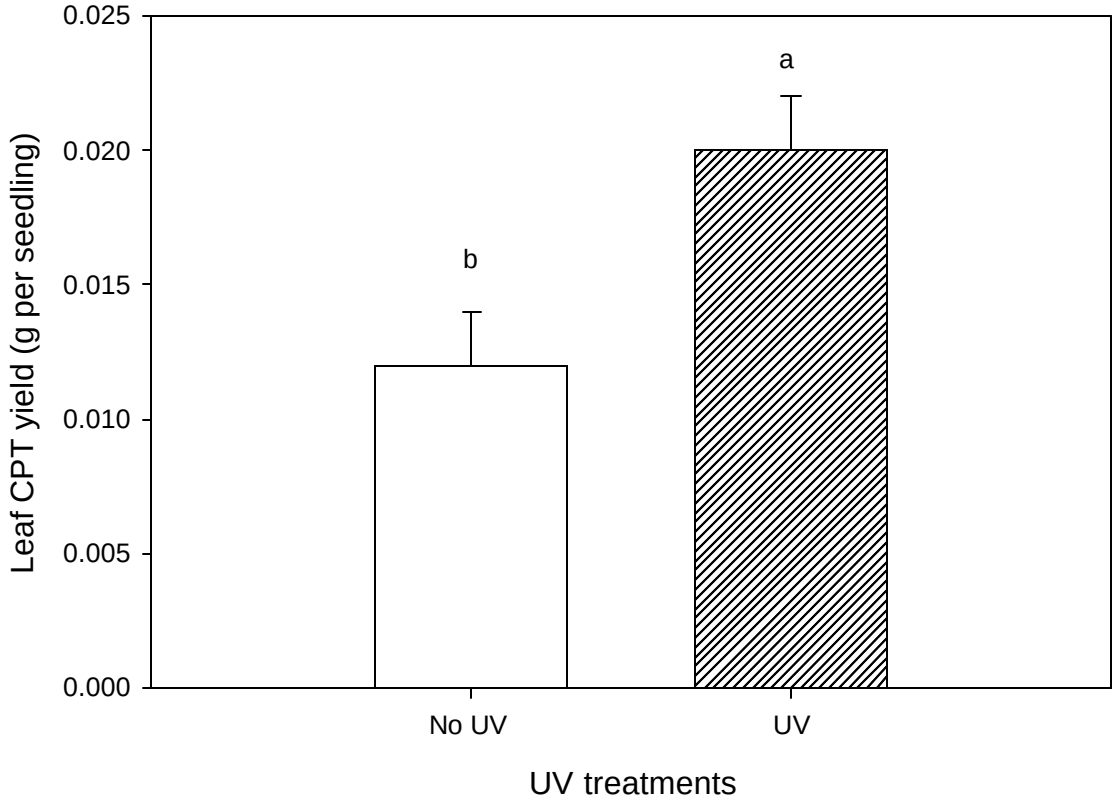

Fig. 3.8. Effect of UV light on CPT yield of *C. acuminata* seedlings in a hydroponic culture system at week 3 of the treatments. Vertical lines above each bar represent standard errors of lsmeans (n=9). Different letters above the bars indicate the significant difference of t tests among the lsmeans at P≤0.05.

Table 3. 9. Covariate analysis with backward variable selections for UV study

Variable	Estimate	Type II SS	F-value	P-value
Intercept	0.05242	0.00241	3.87	0.0577
RSR	0.08634	0.00178	2.86	0.1004
SLW	-0.01545	0.00179	2.87	0.0997

CPT Concentration and Possible Mechanism

In this study, UV light significantly increased leaf CPT concentrations three weeks after exposure. This finding is in agreement with studies on other species. For example, Nicotinamide and trigonelline contents increased in *Pisum sativum* leaves after exposure to UV radiation (Berglund et al. 1996).

Many recent studies were conducted to explore the possible mechanisms of UV light on plant growth and plant secondary metabolism. A possible mechanism may be that UV light activates certain genes and corresponding enzymes responsible for plant secondary metabolism and increases plant secondary metabolite accumulation. For example, UV light was shown to activate the plant defense genes for phytoalexin production in pea (Yamada et al. 1994). The 5' region of an UV light-inducible and elicitor-inducible CHS gene *(chs1)* of soybean has been identified (Wingender et al.1990). The 4-coumarate: CoA ligase (coded by *4cl-1* gene) is a key enzyme in the general phenylpropanoid metabolism in parsley, and the gene *4cl-1* can be transcriptionally activated by stresses, such as pathogen infection and UV-irradiation (Douglas et al. 1991). The tryptophan decarboxylase (coded by *tdc* gene) is a key enzyme in the biosynthesis of terpenoid indole alkaoids (TIAs) in *Catharanthus roseus*. TIAs absorb UV and one of their putative functions in plants includes a role as UV protectants. UV light is found to induce accumulation of several TIAs as well as expression of the *tdc* gene in *C. roseus* and the UV light responsive regions in the promoter of the *tdc* gene from *C. roseus* have been identified (Ouwerkerk et al. 1999a). In *C. rosues*, the *tdc* gene is expressed throughout plant development.

Moreover, *tdc* gene expression is induced by external stress, such as fungal elicitor and UV light. Within the *tdc* promoter sequence, one particular region was identified as the major contributor to basal expression. Another region was shown to be required for induction by fungal elicitors. Binding of nuclear factor, GT-1 with multiple sequences in the promoter was found to be involved in UV-light induced expression (Ouwerkerk et al. 1999b).

Other studies indicate that many enzymes in plant secondary metabolism can be activated by UV light. For example, secondary metabolism was induced in carrot cells by treatment with UV radiation, where incubation of cells in the presence of UV radiation induced the activities of many enzymes, such as phenylalanine ammonia lyase (PAL), CoA-ligase, and chalconisomerase in the phenylpropane and flavonoid metabolism (Broetto and Crocomo 1995). UV light may also have other special effects in plant secondary metabolism to promote the production of secondary metabolite. For example, UV light has been shown to cause the release of an endogenous substance with elicitor activity (Phillips et al. 1992). FMN-mediated coupling of vindoline and catharanthine was stimulated by irradiation with near-ultraviolet light in *C. roseus* seedlings (Hirata et al. 1997).

The lack of interaction between UV and N treatments in our study may suggest that UV light does not promote the utilization of more N resource for CPT biosynthesis. A possible reason might be that UV stress induced more CPT biosynthesis, but N resources both in the N control medium and the N enriched medium were sufficient enough for CPT biosynthesis. In other words, N was not a limiting factor as shown in our N study.

CPT Yield

Final leaf CPT yield was higher in UV treated seedlings than UV un-treated seedlings, where the final CPT yield in leaves depended on two factors: plant biomass and CPT

concentration. Plant biomasses were similar in UV treated seedlings and UV untreated seedlings, but UV treated seedlings had a higher CPT concentration than UV untreated seedlings.

CPT Concentration and Plant Growth, Morphology

This study indicated that plant growth and morphology did not change significantly by the UV treatment, but CPT concentrations did change significantly by the UV treatment. In other words, UV light affected CPT accumulation without affecting plant growth. Meanwhile, CPT concentration changed only after a period of three weeks. Similarly, in *C. acuminata* cell culture, CPT accumulation started at the end of the growth phase (Song et al.1998). The alkaloids tabersonine, ajmalicine, and serpentine were distinctly growth associated, with maximum concentrations in the late exponential or stationary growth phase in *C. roseu*s hairy root culture (Bhadra and Shanks 1997). It is quite possible to use a two-stage process of plant growth, which provides for growth acceleration in vegetative period of development for the purpose of accumulating the largest biomass and then provides for secondary metabolism induction by UV stress at the end of the production process (3 weeks before the harvesting) for secondary metabolite accumulation (Shain 1996).

A slight positive relation was found between CPT concentration and RSR, a similar result as in the nitrogen study. A slight negative relation was found between CPT concentration and SLW. One possible reason for this might be that UV light caused increased CPT concentration but also caused slight damage on leaves, such as leaf distortion and leaf discoloration, as observed in our experiment.

In conclusion, UV light exposure at a peak of 254nm and 1100 $\mu W\ cm^{-2}$ did not affect significantly the growth and morphology of *C. acuminata* seedlings in a hydroponic culture system in a period of three weeks. UV light increased CPT concentration without significantly

affecting plant growth and increased CPT yield three weeks after the exposure. There was no significant interaction between UV light and N treatments on CPT concentration.

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CHAPTER 4. EFFECT OF ACETYLSALICYLIC ACID ON GROWTH, MORPHOLOGY, AND CAMPTOTHECIN ACCUMULATION IN *CAMPTOTHECA ACUMINATA* SEEDLINGS

Introduction

Many abiotic factors affect plant secondary metabolite accumulation; whereas, many biotic factors, especially elicitors [substances produced by invading organisms or damaged plant cells that initiate a complex signaling pathway leading to the activation of defensive response (Taiz and Zerger 1998)], such as peptides, polysaccharide fragments, jasmonic acid, methyl jasmonic acid (MeJa), and acetylsalicylic acid (ASA) are found to trigger the plant defense systems. For example, addition of various concentrations (0.5-20 mM) of ASA to a tumor line of *Catharanthus roseus* cultivated *in vitro* produced remarkable effects on secondary metabolite production, and an increase of 505% in total alkaloids per culture (cells plus liquid medium) was detected (Godoy and Loyola Vargas 1997). This result suggested that ASA could act as a biotic elicitor of plant secondary metabolite production. The possible mechanisms for ASA and other biotic elicitors to induce the plant defense systems though still need to be explored.

Moreover, ASA has a growth retardant property. It was found that in the presence of 0.1 mM ASA in the media, microplant stem growth was retarded in the *in vitro* storage of potato microplants (Lopez Delgado et al. 1998). In another study, stem growth of potato cultured *in vitro* was inhibited significantly by ASA at 0.1-1 mM, which substituted for the growth inhibitor chlorocholine chloride (CCC) (Lopez Delgado and Scott 1997). However, to our knowledge, ASA effect on *C. acuminata* growth has not been reported.

We were interested in addressing these questions: could ASA retard plant growth in *C. acuminata*? Could it trigger plant defense reactions by synthesizing more CPT in plant tissues? The objectives of this study were to examine the effect of ASA on plant growth, morphology,

CPT concentration, and CPT yield, and to define the relationships between plant growth, morphology, and CPT accumulation in *C. acuminata* seedlings.

Materials and Methods

C. acuminata seedlings were propagated in commercial soil plugs from actively expanding shoot tips of *ex vitro* plantlets, with each shoot tips bearing three to four leaves (Liu and Li 2001). These plugs were placed in a hydroponic tray filled with 6 liters of half-strength woody plant medium (WPM, Lloyd and McCown 1981, Sigma Chemicals Inc., St. Louis, MO) solution adjusted to pH 6.5, supplemented with 2 mg l^{-1} indole butyric acid, and enclosed in a mini-chamber. After two to four weeks, the rooted seedlings with similar height (about 7 cm) and the similar leaf number (about 6 leaves) were selected and placed into plastic containers containing 100 ml of half-strength WPM solution. They were allowed to acclimate for one week in chambers prior to the experiment.

To assess the effect of ASA on plant growth, morphology, and CPT accumulation, forty seedlings were chosen and divided equally into four groups, with each group assigned into one of four chambers (blocks). In each chamber, seedlings were assigned randomly to an ASA level of 0, 0.05, 0.1, 0.2, or 0.4 mM. Each ASA treatment was replicated twice within each chamber. These hydroponic seedlings were grown under fluorescent light with 40 µmol $m^{-2}s^{-1}$ light intensity and a photoperiod of 16h and at the room temperature (22-26 °C).

Plant height, weight, leaf number, and blade length of the third leaf from the apex (leaves longer than 1.5 cm were considered to be the first leaves) were used as indicators of plant growth and were measured at the beginning (week 0) and at weeks 1, 2, and 3 of the treatments. Immediately following these measurements, two circular discs (5mm diameter) were punched from each of the 5^{th} and 6^{th} leaves (in the middle of the blade and beside the main vein) on each

seedling, put into a 1.5-ml pre-weighed eppendorf tube, frozen in liquid nitrogen, subsequently freeze-dried, and stored in a refrigerator (2-8 °C) for CPT analysis. The roots, stems, and leaves of each seedling were harvested and weighed to obtaining the root to shoot ratios (RSR) at the end of the 3-week experiment. The RSR and specific leaf weight (SLW, leaf weight per unit leaf area) obtained from the punched leaf discs were used as indicators of plant morphology.

The freeze-dried leaf samples were ground with a pestle in the eppendorf tubes with 25 µl absolute methanol added first to eliminate static electricity. After the grinding, 600 µl methanol was added into the tube. The plant samples in sealed tubes were extracted for CPT on a rotator (at 20 rpm) for at least 16h at the room temperature (22-26 ºC) in the dark. After sample solids settled to the bottom of the tube, the supernatant was filtered through a 0.2 µm filter (Whatman Inc. Fairfield, NJ) and transferred into a new 1.5-ml tube. The filtrate solution was dried by air blowing under a fume hood and reconstituted with 100 µl methanol to achieve a higher concentration of CPT for analysis. Analysis of CPT was performed with a HPLC (Beckman Instruments, Canton, MA) system consisting of a Model 502 autosampler, a Model 125 pump, and a Model 168 photo-diode-array detector as previously described (Liu et al. 1998). CPT concentrations were expressed as percentages of dry weight. CPT yields were obtained from the leaf CPT concentrations and the total dry leaf weights [leaves were used in nondestructive harvestings (Lopez-Meyer et al. 1994)] at the end of the experiment.

Repeated measures analyses were performed for the effects of ASA and time on plant height, weight, leaf number, leaf length, SLW, and leaf CPT concentration. Analyses of variance were performed for the ASA effect on RSR and CPT yield using SAS software. Least square means (lsmeans) were obtained and compared using least significant difference by Student's t test. Plant growth, morphological and chemical data were systematically analyzed with covariate

analysis using backward variable selection in order to find the relationships between plant growth, morphology and CPT concentration and to identify which variables were significantly related to CPT concentration. All tests were regarded as significant at $P \leq 0.05$.

Results

Plant Growth

The effect of ASA addition on height of *C. acuminata* depended on time (Table 4.1). There were no differences in plant height at weeks 0 and 1 of the treatments (Fig. 4.1). However, there were significant differences at weeks 2 and 3. Plant height decreased significantly with increasing ASA concentration, having the least height at the highest ASA level (0.4 mM) and the highest height at the ASA control (0 mM).

Similarly, the effect of ASA addition on *C. acuminata* weight depended on time (Table 4.2). There were no differences in plant weights at week 0 of the treatments (Fig. 4.2). However, there were significant differences at weeks 1, 2 and 3, where plant weight decreased significantly with increasing ASA concentration, having the least weight at the highest ASA level (0.4mM) and the highest weight at the ASA control (0 mM).

There was no significant interaction between ASA and time on leaf number and there was no significant effect of ASA on leaf number (Table 4.3). A slight decrease in leaf number with increasing ASA concentrations at weeks 2 and 3 of the treatments was shown (Fig. 4.3).

The effect of ASA addition on leaf length depended on time (Table 4.4). There were no significant differences in leaf length at the beginning (Fig. 4.4). But there were significant differences in leaf length at weeks 1, 2, and 3 of the ASA treatments, where leaf length decreased dramatically with increasing ASA concentration, and the least leaf length was produced at the highest ASA level (0.4 mM).

Table 4.1. Repeated measures analysis of ASA effect on plant height

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	32	18.92	<.0001
ASA	4	32	2.22	0.0888
Time	3	32	42.31	<.0001
Time*ASA	12	32	2.20	0.0372

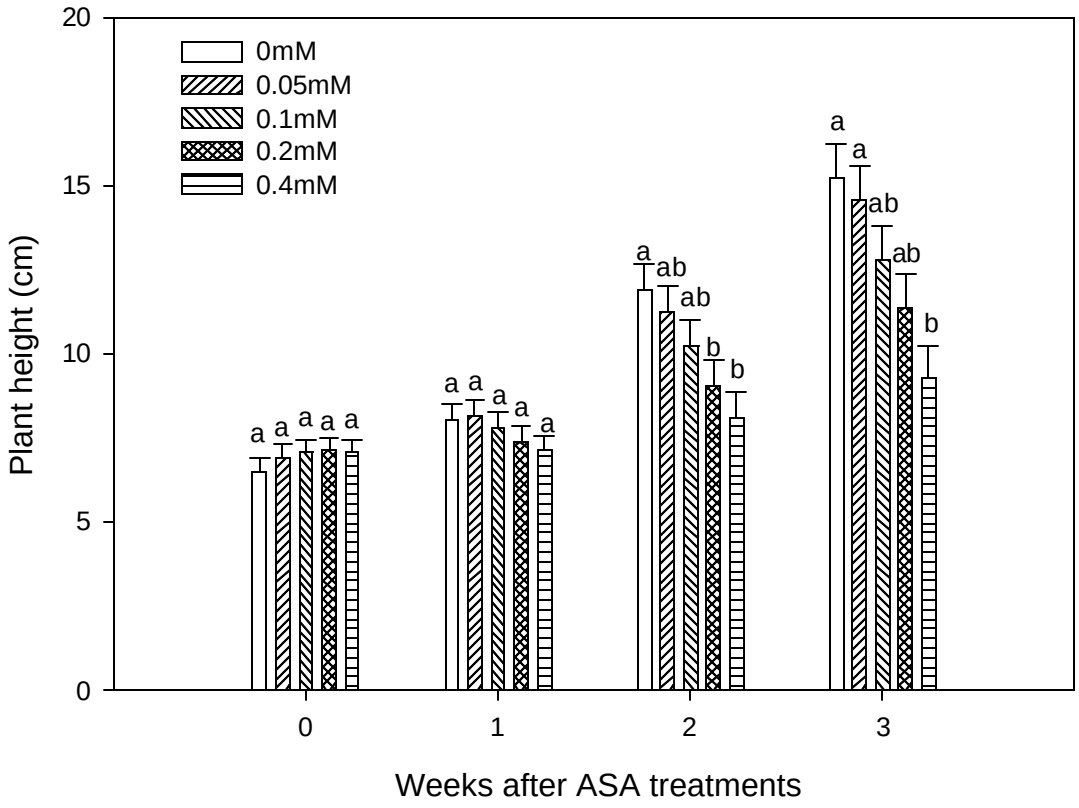

Fig. 4.1. Effect of ASA on height of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant differences of t tests among the lsmeans at $P \leq 0.05$.

Table 4.2. Repeated measures analysis of ASA effect on plant weight

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	32	1.66	0.1945
ASA	4	32	7.16	0.0003
Time	3	32	20.34	<.0001
Time*ASA	12	32	2.31	0.0290

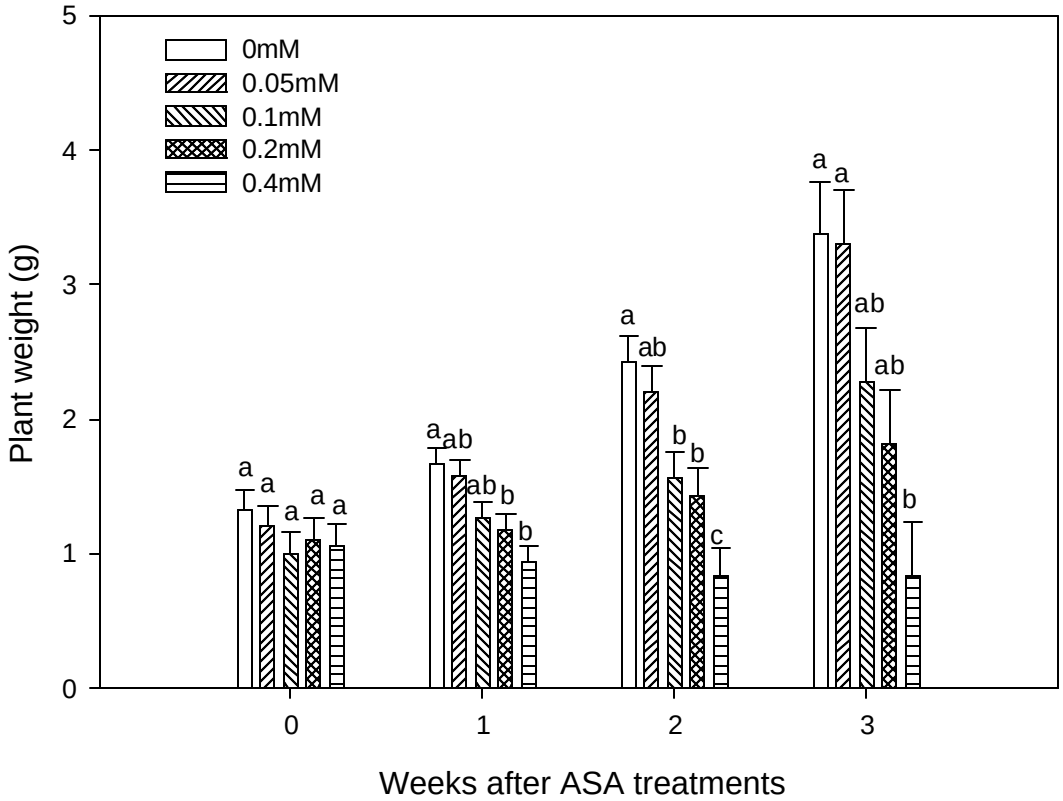

Fig. 4.2. Effect of ASA on weight of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant differences of t tests among the lsmeans at P ≤0.05.

Table 4.3. Repeated measures analysis of ASA effect on leaf number

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	32	31.72	<.0001
ASA	4	32	1.01	0.4178
Time	3	32	53.78	<.0001
Time*ASA	12	32	1.80	0.0920

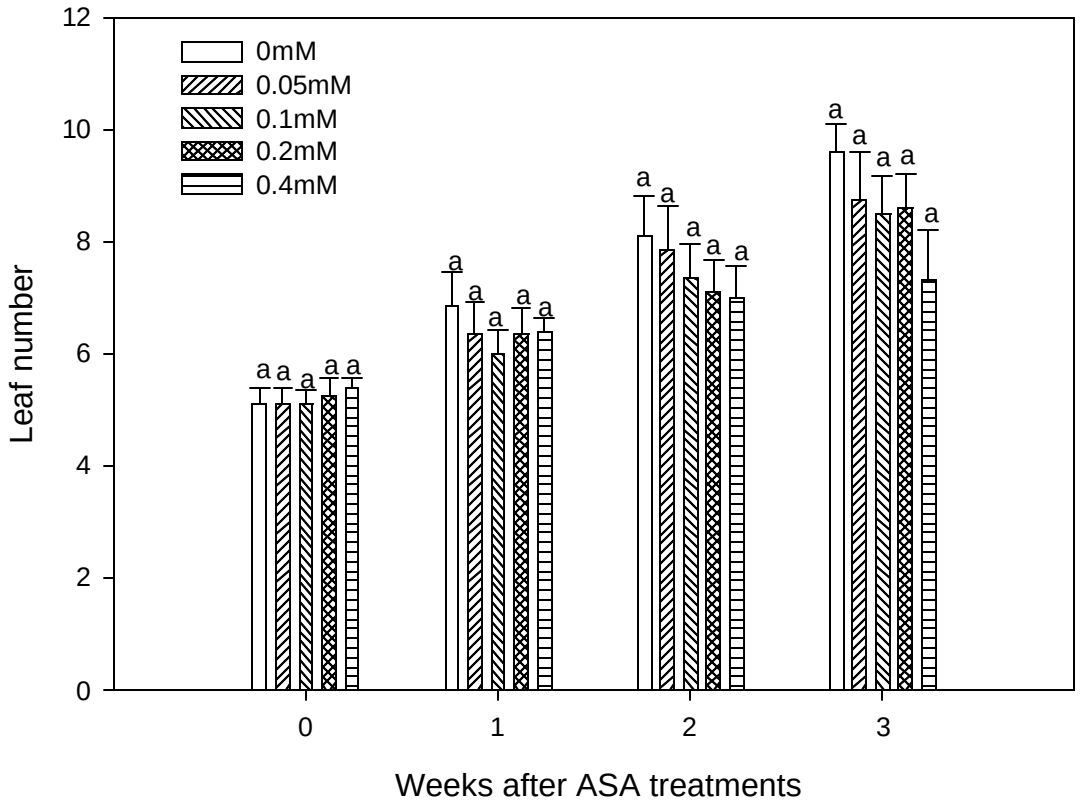

Fig. 4.3. Effect of ASA on leaf number of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant differences of t tests among the lsmeans at P ≤0.05.

Table 4.4. Repeated measures analysis of ASA effect on leaf length

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	32	0.11	0.9525
ASA	4	32	3.20	0.0257
Time	3	32	17.43	<.0001
Time*ASA	12	32	1.99	0.0501

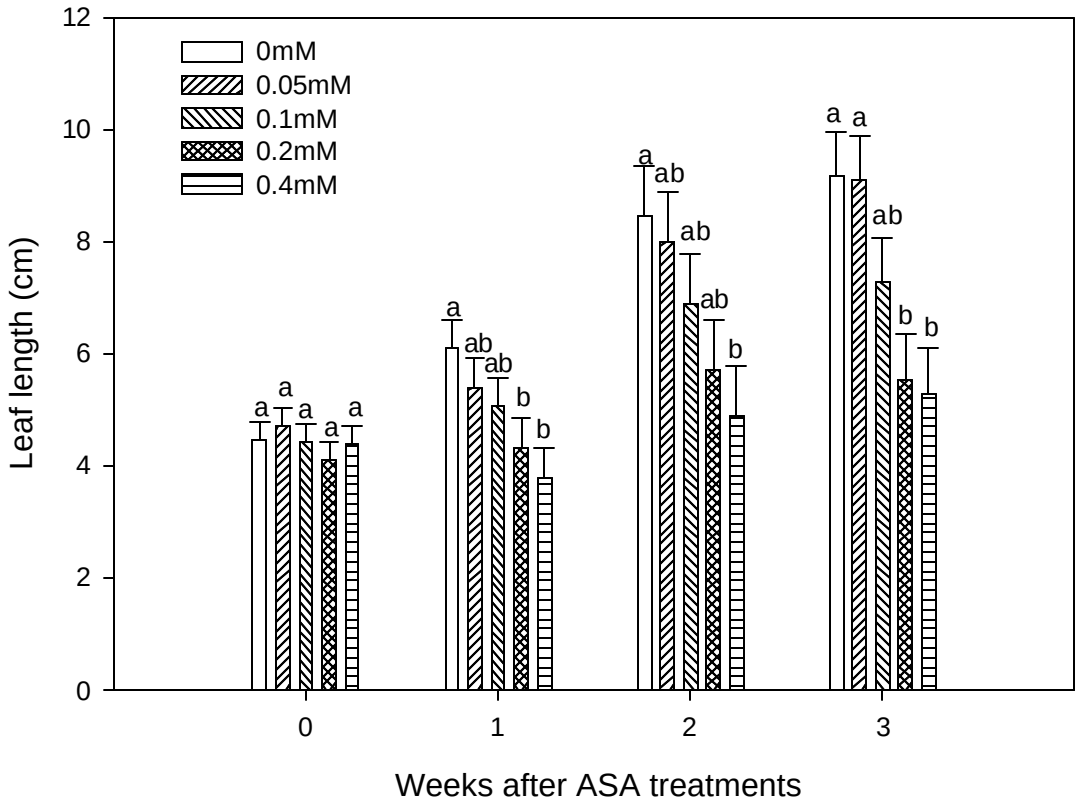

Fig. 4.4. Effect of ASA on leaf length of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant differences of t tests among the lsmeans at $P \leq 0.05$.

Plant Morphologic Changes

ASA addition had no effect on the RSR of *C. acuminata* (Table 4.5). RSR were similar among the ASA treatments at the end of the experiment (Fig. 4.5).

The effect of ASA addition on SLW varied slightly with time (Table 4.6). There were no differences in SLW among all ASA treatments at the beginning (Fig. 4.6). However, there were difference one, two and three weeks after the treatments, where increasing ASA concentration in the media increased SLW and the highest SLW were induced at the highest ASA level (0.4 mM).

CPT Concentration in Leaves and CPT Yield

There was a significant interaction between ASA and time on leaf CPT concentration (Table 4. 7). There were no differences in leaf CPT concentrations among all treatments prior to and one week after the ASA treatments (Fig. 4.7). However, there were significant differences two and three weeks after the ASA treatments, where the highest ASA level (0.4 mM) induced the highest leaf CPT concentration among all ASA levels.

ASA addition had a significant effect on final leaf CPT yield (Table 4.8). CPT yields decreased significantly with increasing ASA concentration, and the highest CPT yield was in the ASA control (Fig. 4.8).

CPT Concentration and Plant Growth, Morphology

Covariate analysis with backward variable selection was used to define the relationships between plant growth, morphology and CPT concentration (Table 4. 9). Among all the variables, such as plant height, weight, leaf number, leaf length, RSR, and SLW, only plant weight was found to relate significantly to CPT concentration, and a negative relation was found between CPT concentration and plant weight.

Table 4.5. Analysis of variance of ASA effect on RSR

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	32	0.71	0.5533
ASA	4	32	1.41	0.2526

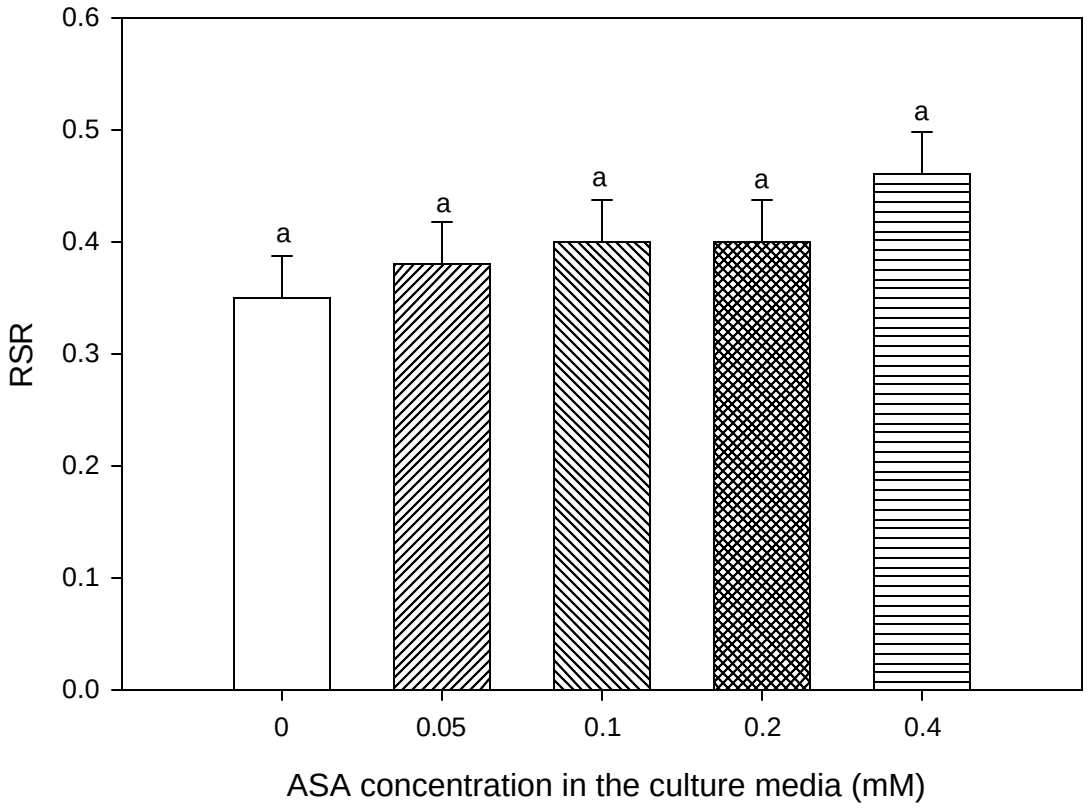

Fig. 4.5. Effect of ASA on RSR of *C. acuminata* seedlings in a hydroponic culture system at week 3 of the treatments. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant differences of t tests among the lsmeans at $P \leq 0.05$.

Table 4.6. Repeated measures analysis of ASA effect on SLW

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	32	3.21	0.0360
ASA	4	32	6.86	0.0004
Time	3	32	9.71	0.0001
Time*ASA	12	32	1.80	0.0920

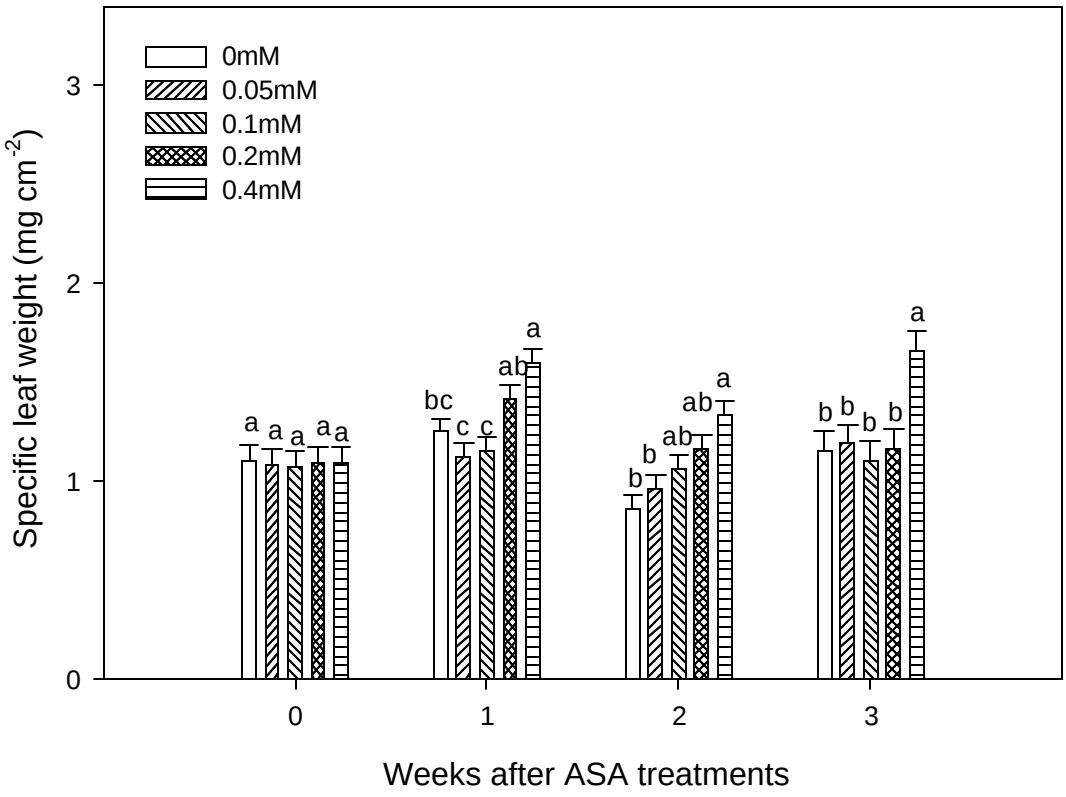

Fig. 4.6. Effect of ASA on SLW of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant differences of t tests among the lsmeans at $P \leq 0.05$.

Table 4.7. Repeated measures analysis of ASA effect on leaf CPT concentration

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	32	1.91	0.1473
ASA	4	32	4.22	0.0074
Time	3	32	15.00	<.0001
Time*ASA	12	32	2.72	0.0118

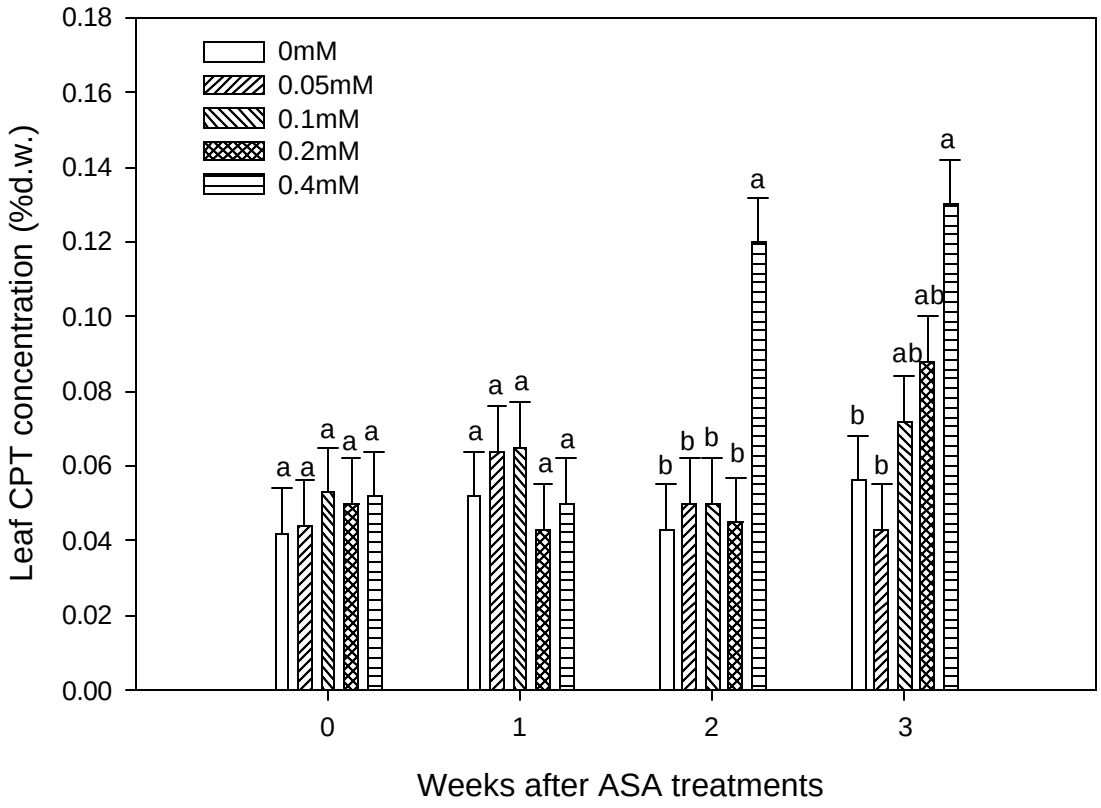

Fig. 4.7. Effect of ASA on leaf CPT concentration of *C. acuminata* seedlings in a hydroponic system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant differences of t tests among the lsmeans at $P \leq 0.05$.

Table 4.8. Repeated measures analysis of ASA effect on CPT yield

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	32	0.76	0.5275
ASA	4	32	3.47	0.0184

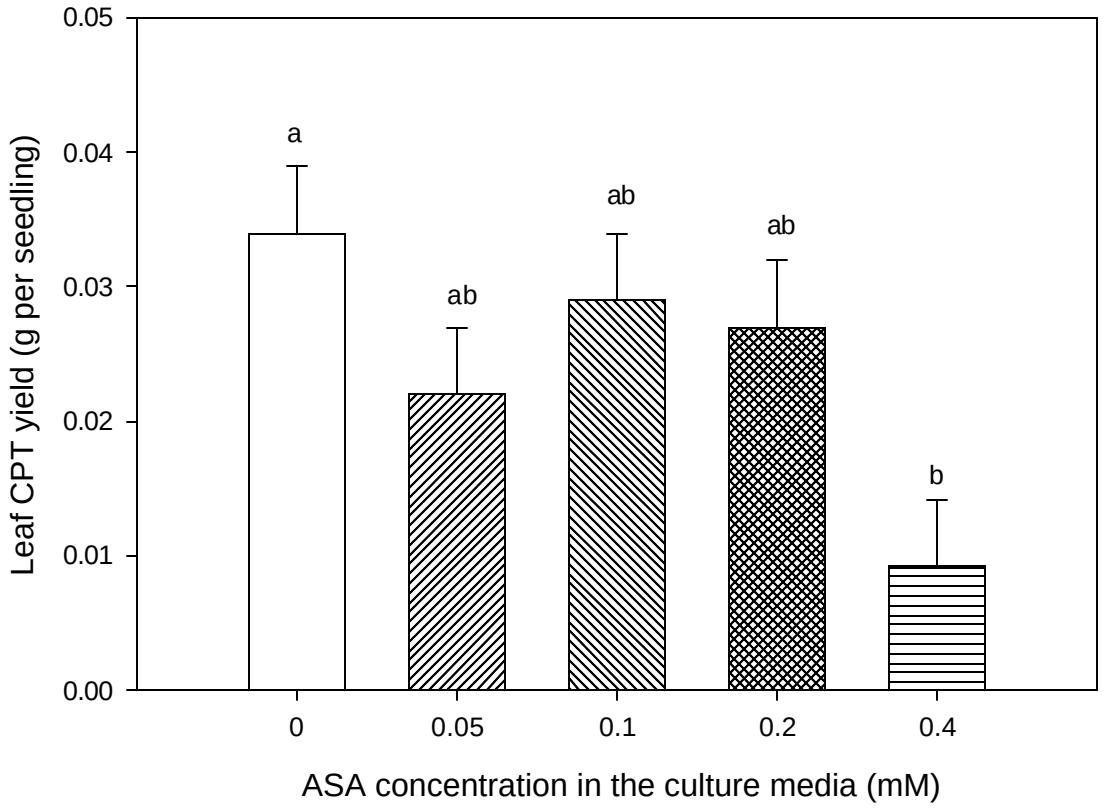

Fig. 4.8. Effect of ASA on CPT yield of *C. acumianta* seedlings in a hydroponic culture system at week 3 of the treatments. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant differences of t tests among the lsmeans at $P \leq 0.05$.

Table 4.9. Covariate analysis with backward variable selection for ASA study

Variable	Estimate	Type II SS	F-value	P-value
Intercept	0.22823	0.38393	80.71	<.0001
Weight	-0.03547	0.07942	16.69	0.0003

Discussion and Conclusions

Plant Growth

In this study, ASA addition decreased plant height, weight, and leaf length of *C. acuminata.* It may suggest that ASA retards the growth of *C. acuminata.* Similarly in the *in vitro* storage of potato microplants, the presence of 0.1 mM ASA in medium retarded microplant stem growth (Lopez Delgado et al. 1998). In another study, stem growth of potato cultured *in vitro* was inhibited significantly by ASA at 0.1-1 mM, as a substitute for the growth inhibitor, chlorocholine chloride (CCC) (Lopez Delgado and Scott 1997). A possible reason for this might be that ASA decreased the levels of endogenous auxins and cytokinins in plant tissues, and thus reduced the elongation and division of plant cells (Hutchinson et al. 1997).

Plant Morphologic Changes

In this study, ASA addition in the media had no effect on RSR. Similarly, the root number and length were not influenced significantly by the ASA treatments in a *in vitro* culture of peach rootstock (Marino and Ventura 1997). These may suggest that plant root growth is not sensitive to ASA treatment. The promotion of shoot regeneration of ASA by inhibition of ethylene production was not found in this study, although it has been reported in other studies (Roustan et al. 1990). This study showed that high ASA concentrations significantly increased the SLW of seedlings, i.e., produced heavier leaves. A possible reason for this might be that the retarded plant growth allowed more photosynthates to accumulate in the plant leaves for defense purpose with increasing fibrous substances.

CPT Concentration and Possible Mechanism

In this study, CPT concentration was not significantly affected by low level of ASA in the range of 0 to 0.2 mM. It is possible that plants at low ASA levels did not accumulate ASA to an effective level to induce the plant defense system, i.e., CPT accumulation.

CPT concentration increased significantly at high ASA level (0.4 mM) as compared with those at low ASA levels. It is possible that at high ASA levels, plants accumulated ASA to a level that activated the plant defense system and produced more CPT. Our finding that high ASA concentration induced significantly more leaf CPT concentration might suggest that ASA could be a stress factor activating the plant defense system and increasing the accumulation of defensive chemicals.

Our results showed that only after a period of two weeks and only at high concentration, did ASA cause significant increase in leaf CPT concentration. This may suggest that ASA affect CPT accumulation in a dose- and time –dependent manner, and a possible ASA accumulation mechanism may be utilized in which ASA accumulates in plant tissues and/or organs to trigger a defense response, in this case, CPT biosynthesis. After accumulated to certain levels, ASA may act on certain enzymes responsible for secondary metabolism. For example, addition of ASA to *Sanguinaria canadensis* cell suspension cultures resulted in an increase in the activity of dihydrobenzophenanthrindine (DHBP) oxidase, an enzyme catalyzing the last step in the biogenesis of the benzophenanthridine alkaloid, sanguinarine. Furthermore, the enzyme-specific activity could be induced in a dose- and time- dependent manner from 4- to 14- fold when cells were treated with ASA (Ignotov et al. 1996). Thus ASA may elicit plant secondary metabolite accumulation by activating the enzymes involved in the biosynthesis of plant secondary metabolites.

CPT Yield

Final leaf CPT yield decreased with increasing ASA concentrations and the highest CPT yield was at no ASA. Final CPT yield in leaves depended on two factors: plant biomass and CPT concentration. Although the highest ASA level produced higher CPT concentration, it produced much lower plant biomass and so it produced a lower CPT yield. On the other hand, the ASA control produced the highest CPT yield, because it produced a similar CPT concentration as low ASA levels but it produced the highest biomass.

CPT Concentration and Plant Growth, Morphology

Covariate analysis showed that among the variables, such as plant height, weight, leaf number, leaf length, RSR, and SLW, only plant weight showed a significant relationship with CPT concentration. The significant negative relationship between CPT concentration and plan weight might suggest that there was a trade-off between plant growth and CPT concentration (Bryant et al. 1983). A possible explanation might be that CPT concentration increased at the cost of decreasing plant growth.

In conclusion, ASA addition reduced plant height, weight, and leaf length, but increased SLW. ASA addition at a high concentration induced elevated leaf CPT concentrations. However, CPT yields decreased with increasing ASA levels. A significant negative relationship was found between CPT concentration and plant weight under the ASA treatments.

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CHAPTER 5. EFFECT OF PLANT HORMONES BA AND NAA ON GROWTH, MORPHOLOGY, AND CAMPTOTHECIN ACCUMULATION IN *CAMPTOTHECA ACUMINATA* SEEDLINGS

Introduction

The plant hormones, cytokinins, such as benzyl adenine (BA), zeatin, and kinetin, have important physiolo gical effects on plant growth and morphology. For example, they promote shoot formation and lateral bud expansion, and delay leaf senescence through their functions in promoting cell division and cell differentiation. Cytokinins are also shown to be involved in plant secondary metabolism. For example, cytokinins were found to stimulate alkaloid biosynthesis in cell lines of *Catharanthus ro*seus (Decendit et al. 1992).

The plant hormones, auxins, such as naphthalene acetic acid (NAA) and indole acetic acid (IAA), also have important physiological effects on plant growth and morphology. They promote apical dominance, lateral and adventitious root formation, stem elongation and leaf elongation by a mechanism of promoting cell elongation. They also play roles in regulating plant secondary metabolism. For example, in the transformed root cultures of *Hyoscyamus muticus*, the growth of the roots was not affected by NAA, but accumulation of alkaloid was doubled compared with that of the roots growing in the absence of auxin, i.e., alkaloid accumulation had been increased by exogenous auxins without influencing growth (Vanhala et al. 1998). However, in *C. roseus* cell suspensions, omission of 2,4-D from the medium resulted in an increased alkaloid accumulation (Avry et al. 1994).

We hypothesized that cytokinins and auxins affect CPT accumulation. Our literature reviews found that different types of cytokinins affect plant secondary metabolism in similar ways (Coulillerot et al. 1996).BA is relatively effective and cheap among cytokinins. Different types of auxins affect plant secondary metabolism metabolism in similar ways (Goddijn et al.

1992). NAA is relatively effective and cheap among auxins. The objectives of this study were to evaluate the effects of BA and NAA on plant growth, morphology, CPT accumulation, and CPT yield in *C. acuminata*, and to find the relationships between plant growth, morphology and CPT accumulation.

Materials and Methods

C. acuminata seedlings were propagated in commercial soil plugs from actively growing shoot tips of *ex vitro* plants, with the shoot tips bearing three to four leaves (Liu and Li 2001). These plugs were placed in a hydroponics tray filled with 6 liters of half-strength woody plant medium (WPM, Lloyd and McCown 1981, Sigma Chemicals Inc., St. Louis, MO) solution adjusted to pH 6.5 and supplemented with 2 mg l^{-1} indole butyric acid, and enclosed in a mini-chamber. After two to four weeks, the rooted seedlings with similar height (about 10 cm) and similar leaf numbers (about 6 leaves) were selected and placed into plastic containers containing 100 ml half-strength WPM solution. Seedlings were allowed to acclimate for one week in the chambers prior to the experiment.

Two separate experiments were conducted to test for BA and NAA effects on plant growth, morphology, and CPT accumulation. In the BA study, thirty-two seedlings were chosen and divided into four groups, with each group assigned into one of four chambers (blocks). In each chamber, seedlings were assigned randomly a BA level of 0, 0.3, 1, or 3 mg l^{-1} (or 0, 1.32, 4.4, and 13.2 µM, because mg l^{-1} unit was easier to handle in lab manipulation, hereafter we used this unit). BA applications were done as follows. A 3.2 liters half-strength WPM solution was prepared and divided equally into four flasks. BA stock solutions were then added variably to four flasks to arrive at 0, 0.3, 1, or 3 mg l^{-1} BA in solution. A 100 ml aliquot of each of these solutions was then dispensed into a seedling container. Each BA treatment was replicated twice

within each of 4 chambers. Seedlings were grown under fluorescent lights providing 40 μmol $m^{-2}s^{-1}$ light intensity and 16h photoperiod and at the room temperature (22-26 °C). The NAA study was performed in a similar manner as the BA study, except that 40 seedlings were selected and five levels of NAA at 0, 0.5, 1, 2, or 4 mg l^{-1} (or 0, 2.7, 5.4, 10.8, or 21.6 μM) were used.

Plant height, weight, leaf number, and blade length of the third leaf from the apex (leaves longer than 1.5 cm were considered to be the first leaves) were used as indicators of plant growth and were measured at the beginning (week 0) and weeks 1, 2, and 3 of the treatment. Immediately following these measurements, two circular discs (5mm diameter) were punched from each of the 5^{th} and 6^{th} leaves (in the middle of the blade and beside the main vein) on each seedling, put into a 1.5-ml pre-weighed eppendorf tube, frozen in liquid nitrogen, subsequently freeze-dried, and stored in a refrigerator (2-8 °C) for CPT analysis. Roots, stems, and leaves of each seedling were harvested and weighed to obtain the root to shoot ratios (RSR) at the end of the 3-week experiment. The RSR and specific leaf weight (SLW, leaf weight per unit leaf area) obtained from the punched leaf discs were used as indicators of plant morphology.

The freeze-dried leaf samples were ground with a pestle in the eppendorf tubes with 25 μl absolute methanol added first to eliminate static electricity. After the grinding, 800 μl methanol was added into each tube. Plant samples were extracted for CPT on a rotator for at least 16h at the room temperature (22-26 °C) in the dark. After sample solids settled to the bottom of the tube, the supernatant was filtered through a 0.2 μm filter (Whatman Inc. Fairfield, NJ) and transferred into a new 1.5-ml tube. The filtrate solution was dried by air blowing under a hood and reconstituted with 100 μl methanol to obtain a higher accumulation of CPT for analysis. Analysis of CPT was performed with a HPLC system (Beckman Instruments, Canton, MA) consisting of a Model 502 autosampler, a Model 125 pump, and a Model 168 photo-diode-array

detector as described previously (Liu et al. 1998). CPT accumulation was expressed as a percentage of dry weight. CPT yield was obtained from the leaf CPT accumulation and the total dry leaf weight [leaves were used in nondestructive harvestings (Lopez-Meyer et al. 1994)] at the end of the experiment.

Repeated measures analyses were performed for the effects of BA, NAA, and time on plant height, weight, leaf number, leaf length, SLW, and leaf CPT accumulation. Analyses of variance were performed for the effects of BA and NAA on RSR and CPT yield using SAS software Mixed procedures. Least square means (lsmeans) were obtained and compared using least significant difference by Student's t test. Plant growth, morphological, and chemical data were systematically analyzed with covariate analysis with backward variable selection in order to find the relationships between plant growth, morphology and CPT accumulation, and to identify which variables were related significantly to CPT accumulation. All tests were regarded as significant at $P \leq 0.05$.

Results

Plant Growth

The effect of BA on plant height of *C. acuminata* depended on time (Table 5.1-1). BA applications ranging from 0 to 3 mg l^{-1} had no effect on plant height at weeks 0, 1, and 2 of the treatments, whereas plant height declined significantly with increasing accumulation of BA at week 3 of the experiment (Fig. 5.1-1). The effect of NAA on plant height also depended on time (Table 5.1-2). NAA applications ranging from 0 to 4 mg l^{-1} had no effect on plant height at weeks 0 and 1 of the treatments, whereas plant height declined significantly with NAA supplements in comparison with the NAA control (Fig. 5.1-2).

Table 5.1-1. Repeated measures analysis of BA effect on plant height

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	25	14.79	<.0001
BA	3	25	1.60	0.2143
Time	3	25	211.35	<.0001
Time*BA	9	25	4.78	0.0009

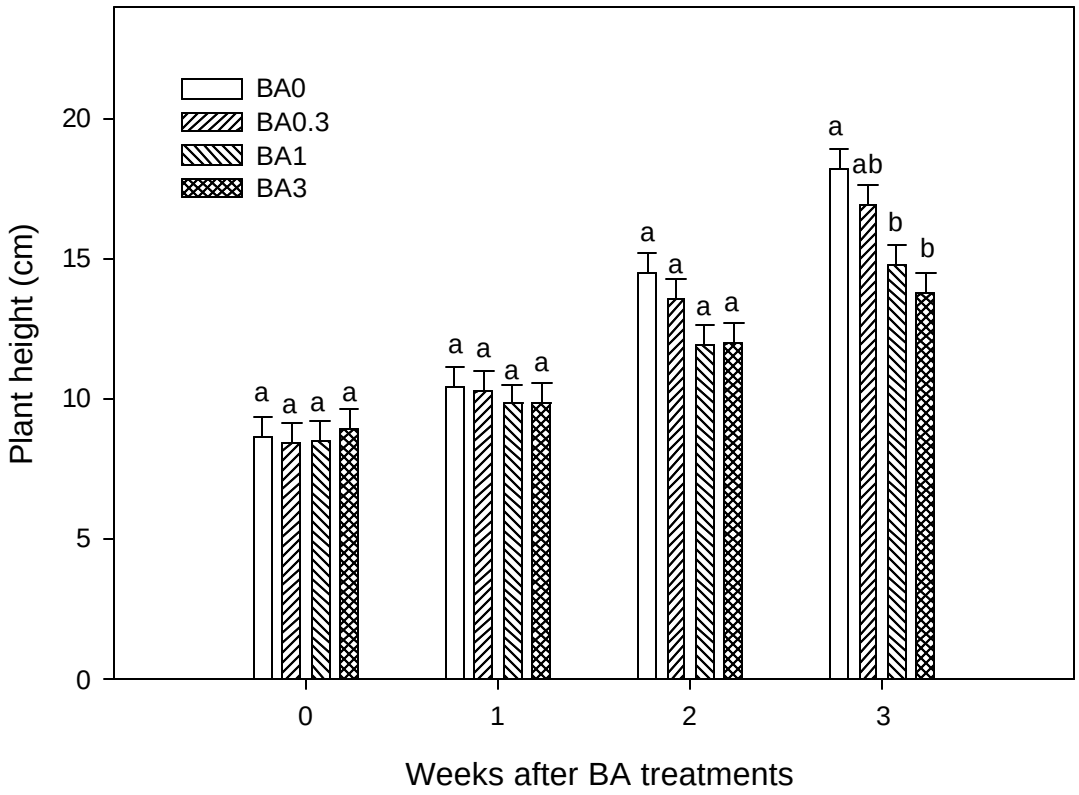

Fig. 5.1-1. Effect of BA on height of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant difference of the t tests among the lsmeans at $P \leq 0.05$.

Table 5.1-2. Repeated measures analysis of NAA effect on plant height

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	32	11.21	<.0001
NAA	4	32	7.04	0.0003
Time	3	32	53.77	<.0001
Time*NAA	12	32	10.88	<.0001

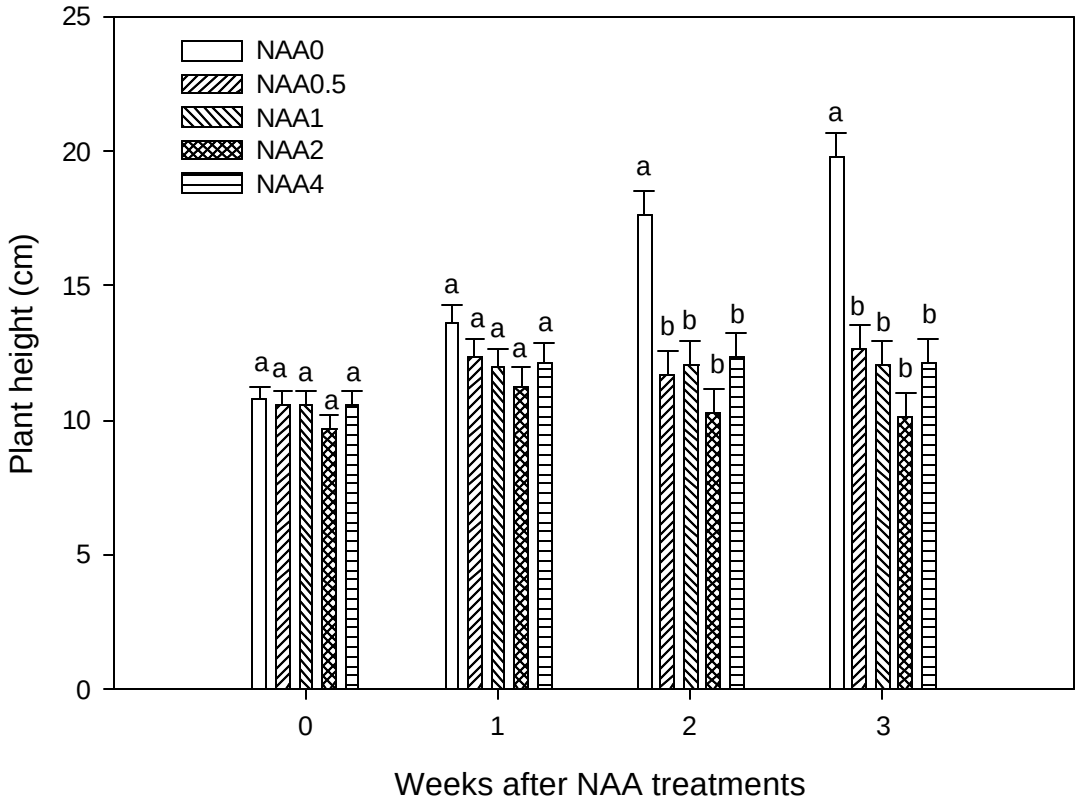

Fig. 5.1-2. Effect of NAA on height of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate significant difference of t tests among the lsmeans at P ≤0.05.

There was no significant interaction between BA and time on plant weight and BA applications in the solution had no effects on plant weight (Table 5.2-1, Fig. 5.2-1). There was a significant interaction between NAA and time on plant weight (Table 5.2-2). There were no differences in plant weight at week 0, but increasing NAA accumulations from 0 to 4 mg l^{-1} significantly decreased plant weight at weeks 1, 2, and 3 of the treatments (Fig. 5.2-2).

There was no significant interaction between BA and time on leaf number and BA had no effect on leaf number (Table 5.3-1). Leaf numbers were similar among all BA treatments at weeks 0, 1, and 2, but were slightly different at week 3, where increasing BA levels in the media decreased leaf number (Fig. 5.3-1). The effect of NAA applications on leaf number depended on time (Table 5.3-2). There were no differences in leaf numbers at weeks 0 and 1, but increasing NAA levels led to a significant decline in leaf numbers at weeks 2 and 3 (Fig. 5.3-2).

There was no significant interaction between BA and time on leaf length, and there was no significant effect of BA on leaf length (Table 5.4-1). But there was a slight decrease in leaf length with increasing BA accumulation at week 3 (Fig. 5.4-1). The effect of NAA on leaf length depended on time (Table 5.4-2). NAA applications from 0 to 4 mg l^{-1} had no effect on leaf length at weeks 0 and 1, but had significant effect on leaf length at weeks 2 and 3 (Fig 5.4-2). NAA applications from 0.5 to 4 mg l^{-1} significantly reduced leaf length compared with the control.

Plant Morphological Changes

BA application significantly affected the RSR (Table 5.5-1). Increasing BA accumulation led to increased RSR at week 3 of the treatments (Fig. 5.5-1). Similarly, NAA application significantly affected the RSR (Table 5.5-2), and NAA application resulted in increased RSR three weeks after the treatments (Fig. 5.5-2).

Table 5.2-1. Repeated measures analysis of BA effect on plant weight

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	25	7.52	0.0010
BA	3	25	1.76	0.1798
Time	3	25	60.00	<.0001
Time*BA	9	25	0.61	0.7764

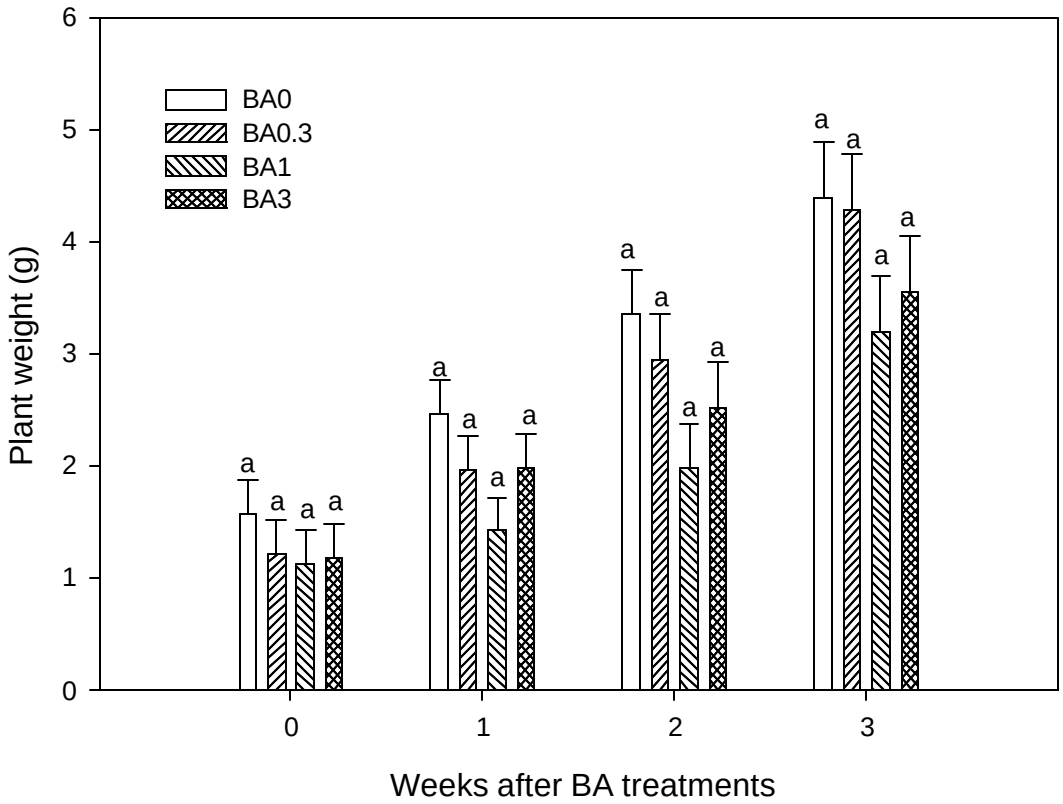

Fig. 5.2-1. Effect of BA on weight of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant difference of the t test among the lsmeans at P ≤0.05.

Table 5.2-2. Repeated measures analysis of NAA effect on plant weight

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	32	2.06	0.1246
NAA	4	32	5.47	0.0018
Time	3	32	45.56	<.0001
Time*NAA	12	32	4.53	0.0003

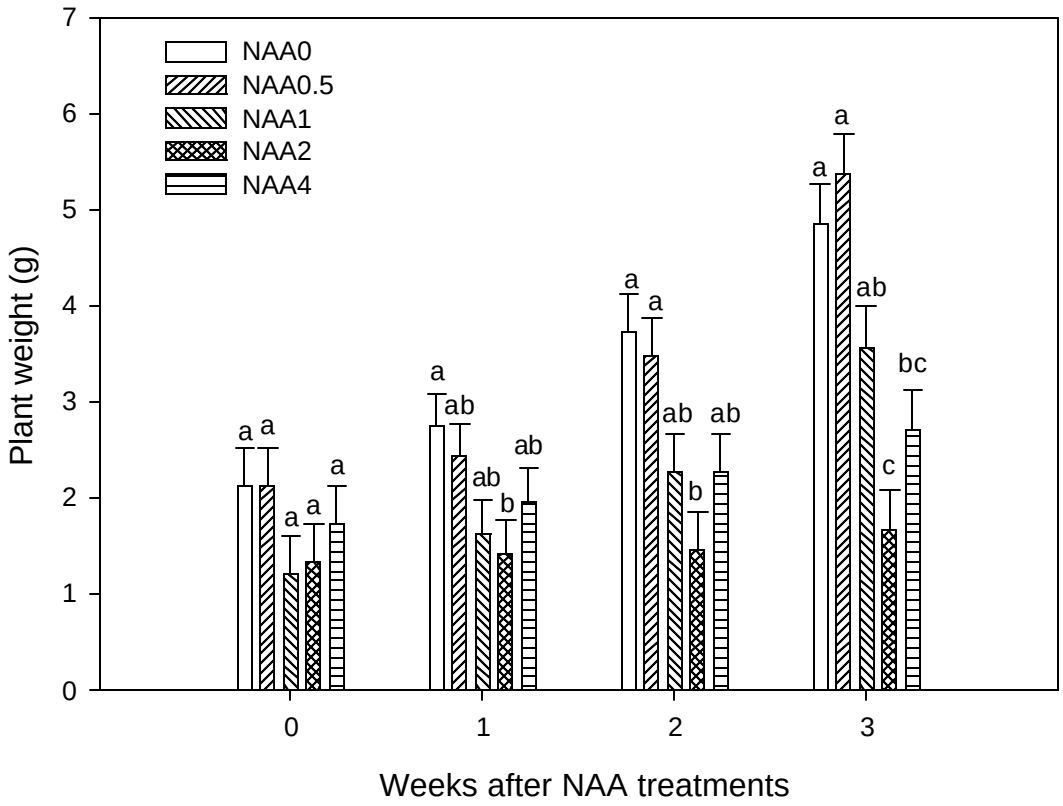

Fig. 5.2-2. Effect of NAA on weight of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate significant difference of t test among lsmeans at P ≤0.05

Table 5.3-1. Repeated measures analysis of BA effect on leaf number

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	25	17.82	<.0001
BA	3	25	1.72	0.1878
Time	3	25	257.48	<.0001
Time*BA	9	25	1.64	0.1572

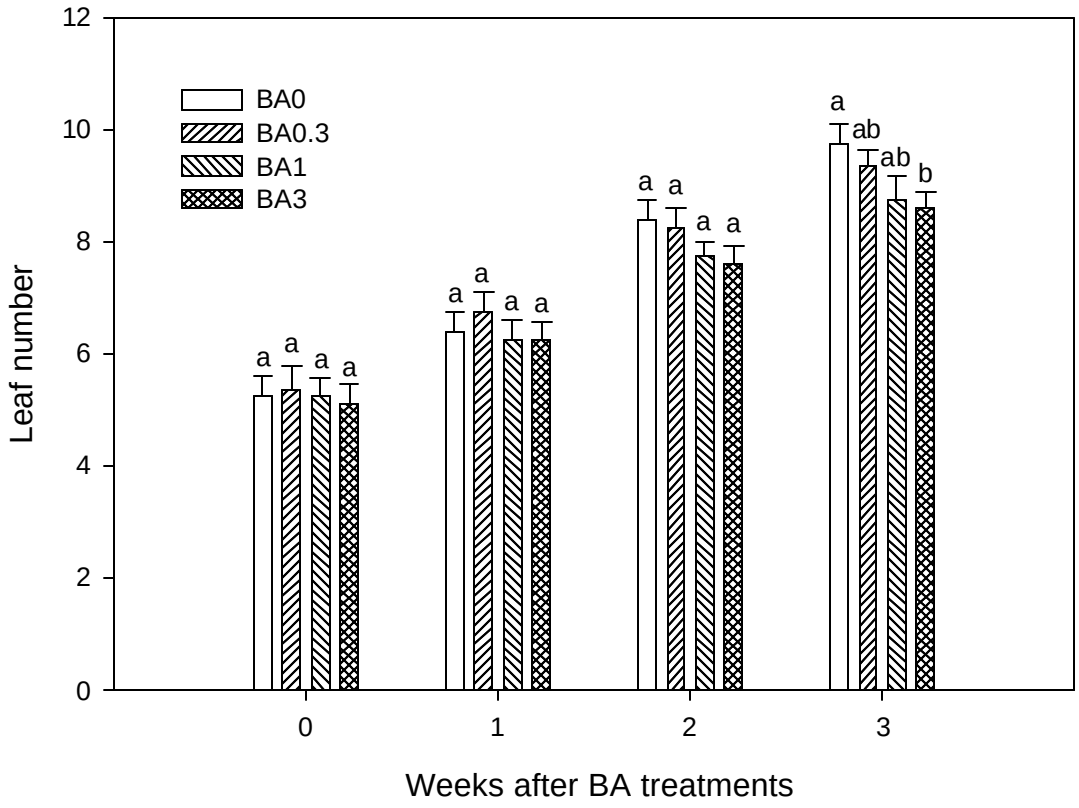

Fig. 5.3-1. Effect of BA on leaf number of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant difference of the t test among the lsmeans at P ≤0.05.

Table 5.3-2. Repeated measures analysis of NAA effect on leaf number

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	32	992.48	<.0001
NAA	4	32	13.38	<.0001
Time	3	32	83.97	<.0001
Time*NAA	12	32	7.73	<.0001

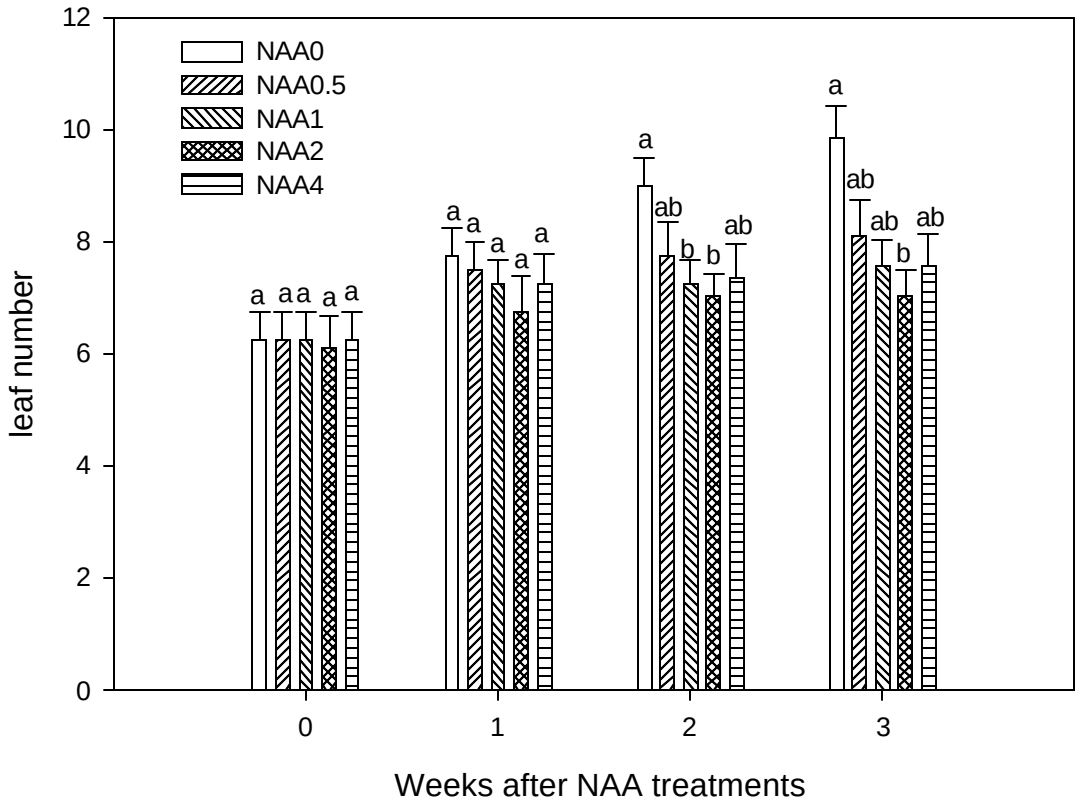

Fig. 5.3-2. Effect of NAA on leaf number of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate significant difference of t test among the lsmeans at P ≤0.05.

Table 5.4 -1. Repeated measures analysis of BA effect on leaf length

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	25	2.22	0.1112
BA	3	25	0.93	0.4390
Time	3	25	52.14	<.0001
Time*BA	9	25	1.83	0.1133

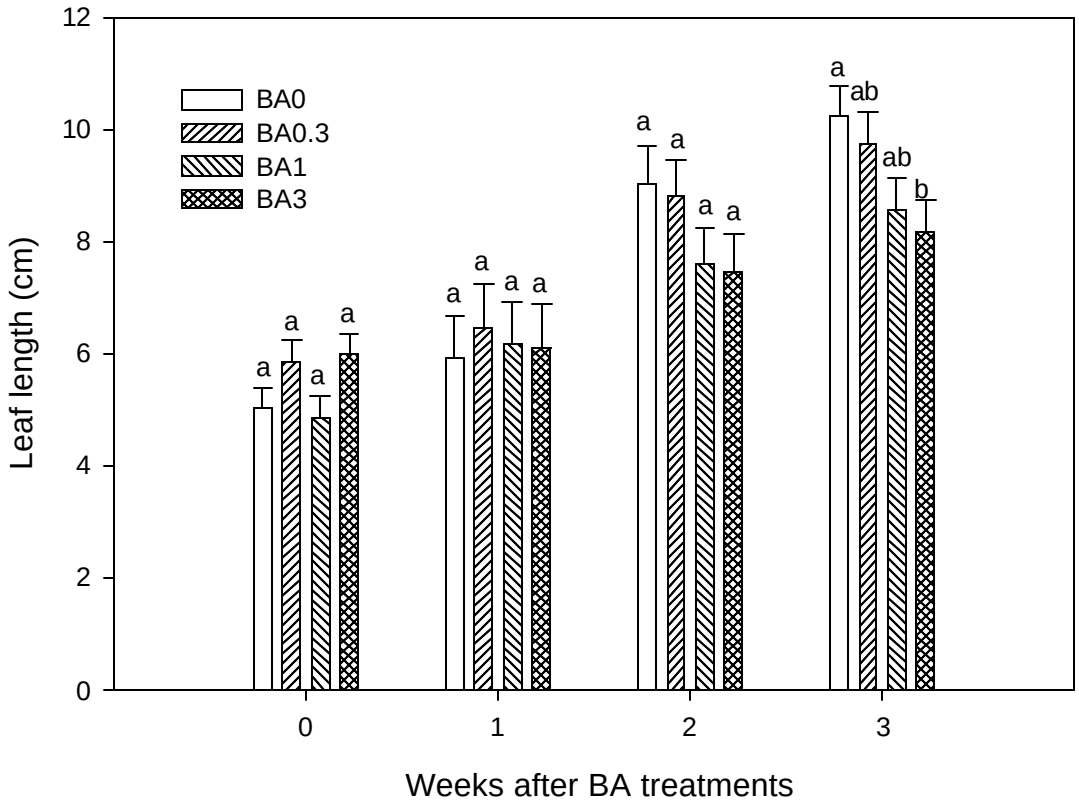

Fig. 5.4-1. Effect of BA on leaf length of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate significant difference of the t test among the lsmeans at P ≤0.05.

Table 5.4 -2. Repeated measures analysis of NAA effect on leaf length

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	32	16.59	<.0001
NAA	4	32	1.75	0.1633
Time	3	32	15.06	<.0001
Time*NAA	12	32	2.60	0.0155

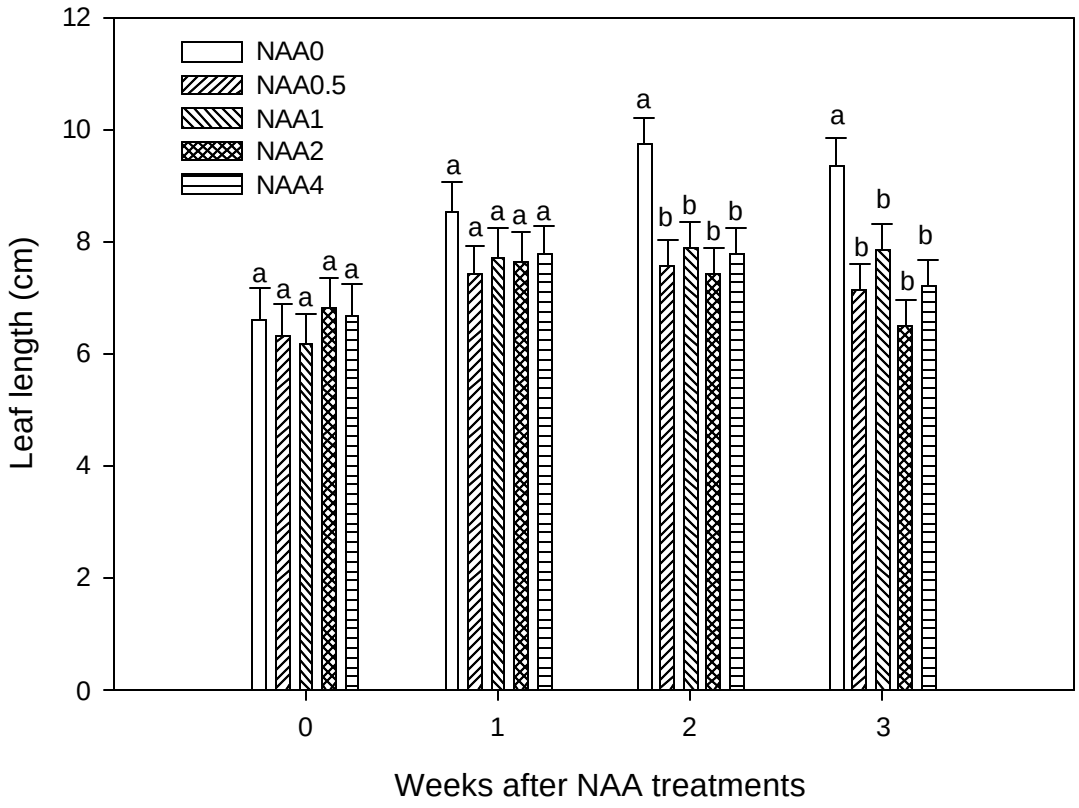

Fig. 5.4-2. Effect of NAA on leaf length of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate significant difference of t tests among the lsmeans at P ≤0.05.

Table 5.5 -1. Analysis of variance of BA effect on RSR

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	25	4.06	0.0177
BA	3	25	20.79	<.0001

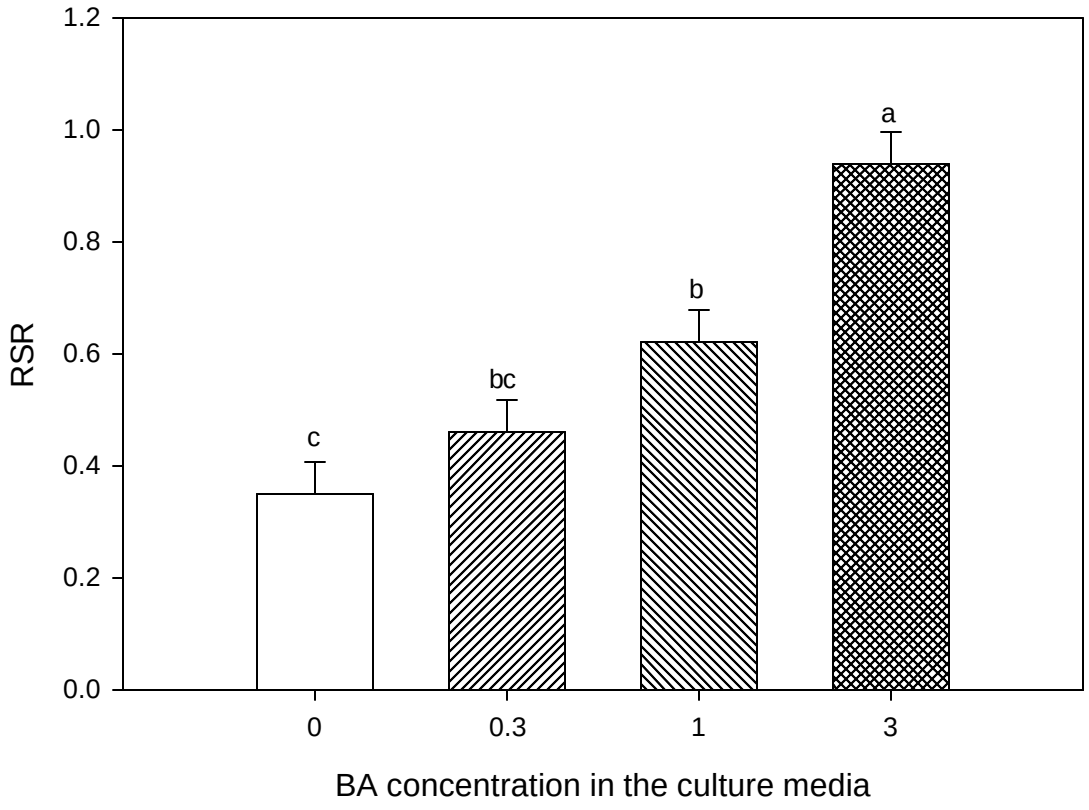

Fig. 5.5-1. Effect of BA on RSR of *C. acuminata* seedlings in a hydroponic culture system at week 3 of the treatments. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant difference of the t test among the lsmeans at $P \leq 0.05$.

Table 5.5-2. Analysis of variance of NAA effect on RSR

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	32	0.81	0.5000
NAA	4	32	6.11	0.0009

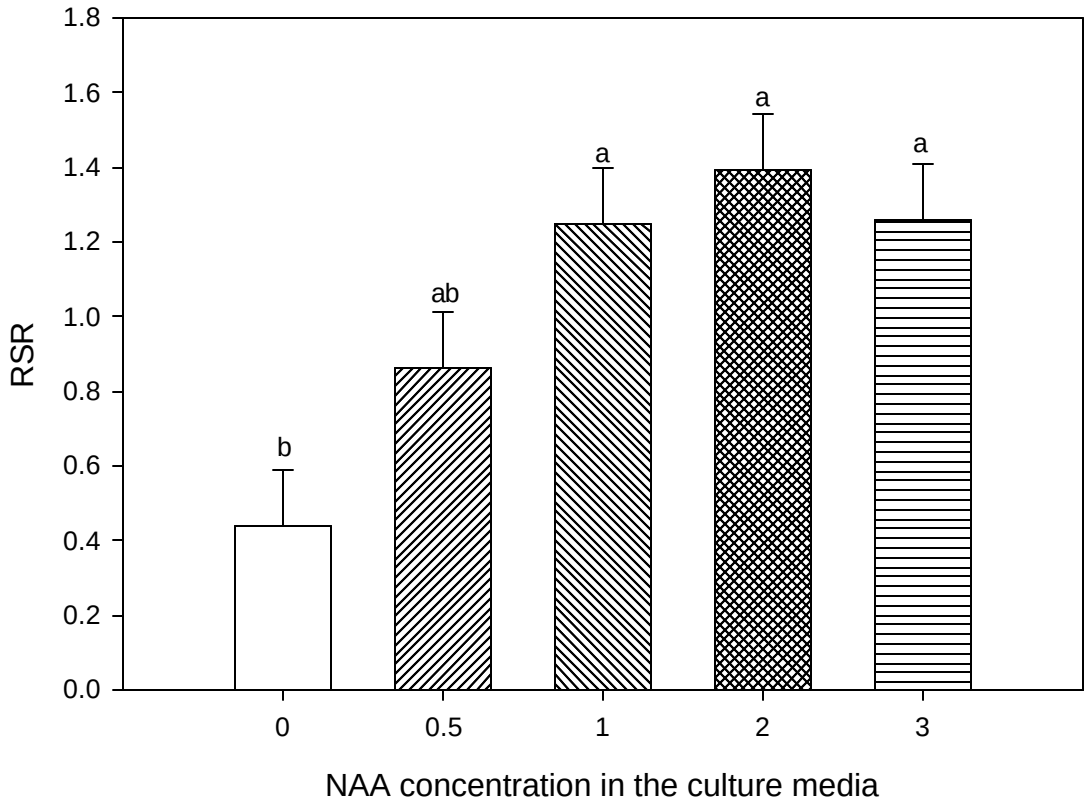

Fig. 5.5-2. Effect of NAA on RSR of *C. acuminata* seedlings in a hydroponic culture system at week 3 of the treatments. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant difference of the t tests among the lsmeans at $P \leq 0.05$.

There was no significant interaction between BA and time on SLW, and there was also no significant effect of BA on SLW (Table 5.6-1). But SLW decreased slightly with increasing BA accumulation at week 3 of the BA treatments (Fig. 5.6-1). There was a significant interaction between NAA and time on SLW (Table 5.6-2). There were no differences in SLW among all NAA treatments at weeks 0, 1, and 2, but SLW was significantly reduced in NAA applications compared with the control at week 3 (Fig. 5.6-2).

CPT Accumulations in Leaves and CPT Yield

There was a significant interaction between BA and time on leaf CPT accumulation (Table 5.7-1). Prior to the BA treatments, there were no differences in leaf CPT accumulations among the treatments, indicating homogeneous experimental materials (Fig. 5.7-1). There were no differences in leaf CPT accumulations one and two weeks after the BA treatments. However, leaf CPT accumulations were significantly higher in seedlings treated with high levels of BA three weeks after the treatments.

There was no significant interaction between NAA and time on leaf CPT accumulation and there was no significant effect of NAA on leaf CPT accumulation (Table 5.7-2). Prior to the NAA treatments, there were no differences in leaf CPT accumulations among the treatments, and NAA application did not change leaf CPT accumulations throughout the three weeks of the experiment (Fig. 5.7-2).

BA application had no effect on CPT yield (Table 5.8-1), and BA applications from 0 to 3 mg l^{-1} produced similar CPT yield (Fig 5.8-1). On the other hand, NAA application had a significant effect on CPT yield (Table 5.8-2). Increasing NAA accumulations significantly reduced CPT yield, and the highest CPT yield was in the NAA control (Fig 5.8-2).

Table 5.6-1. Repeated measures analysis of BA effect on SLW

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	25	1.31	0.2948
BA	3	25	2.50	0.0826
Time	3	25	0.83	0.4922
Time*BA	9	25	1.15	0.3660

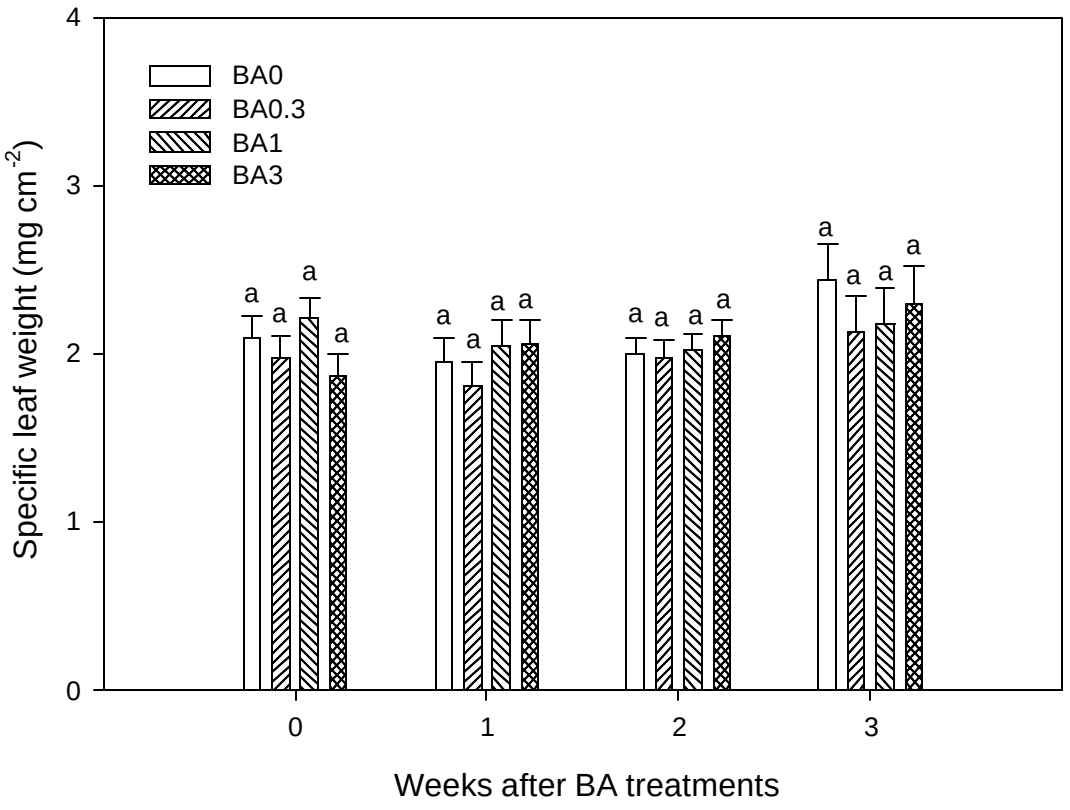

Fig. 5.6-1. Effect of BA on SLW of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant difference of the t tests among the lsmeans at P ≤0.05.

Table 5.6-2. Repeated measures analysis of NAA effect on SLW

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	32	4.13	0.0139
NAA	4	32	3.23	0.0247
Time	3	32	132.32	<.0001
Time*NAA	12	32	4.60	0.0003

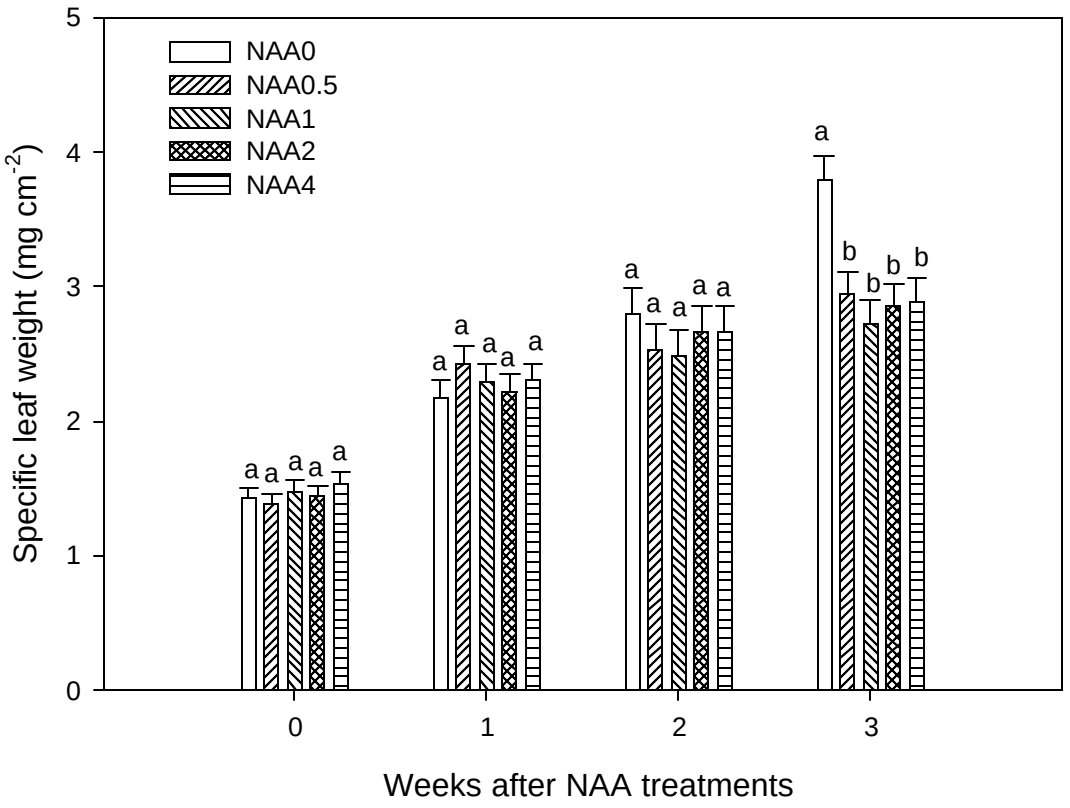

Fig. 5.6-2. Effect of NAA on SLW of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant difference of the t tests among the lsmeans at P ≤0.05.

Table 5.7-1. Repeated measures analysis of BA effect on leaf CPT accumulation

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	25	1.18	0.3373
BA	3	25	0.61	0.6135
Time	3	25	4.10	0.0169
Time*BA	9	25	2.21	0.0506

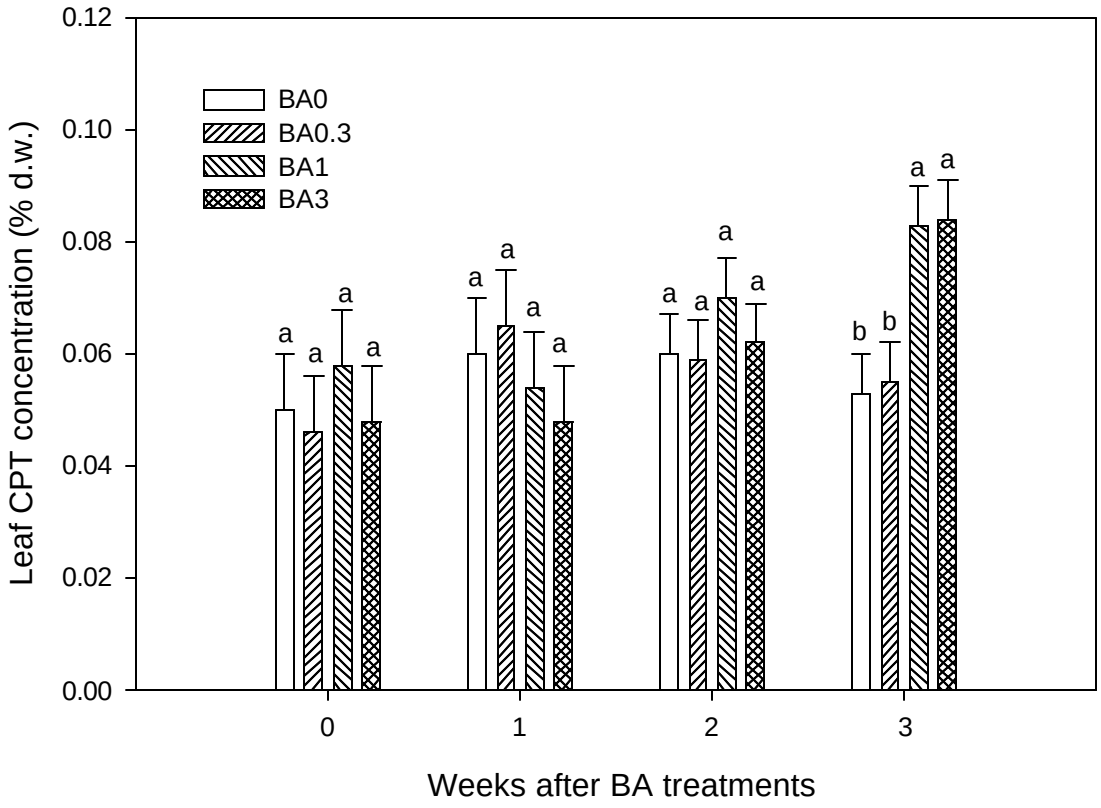

Fig. 5.7-1. Effect of BA on leaf CPT accumulation of *C. acuminata* seedlings in a hydroponic system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant difference of t tests among the lsmeans at $P \leq 0.05$.

Table 5.7-2. Repeated measures analysis of NAA effect on leaf CPT accumulation

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	32	2.48	0.0570
NAA	4	32	0.01	0.9997
Time	3	32	1.03	0.3923
Time*NAA	12	32	0.16	0.9991

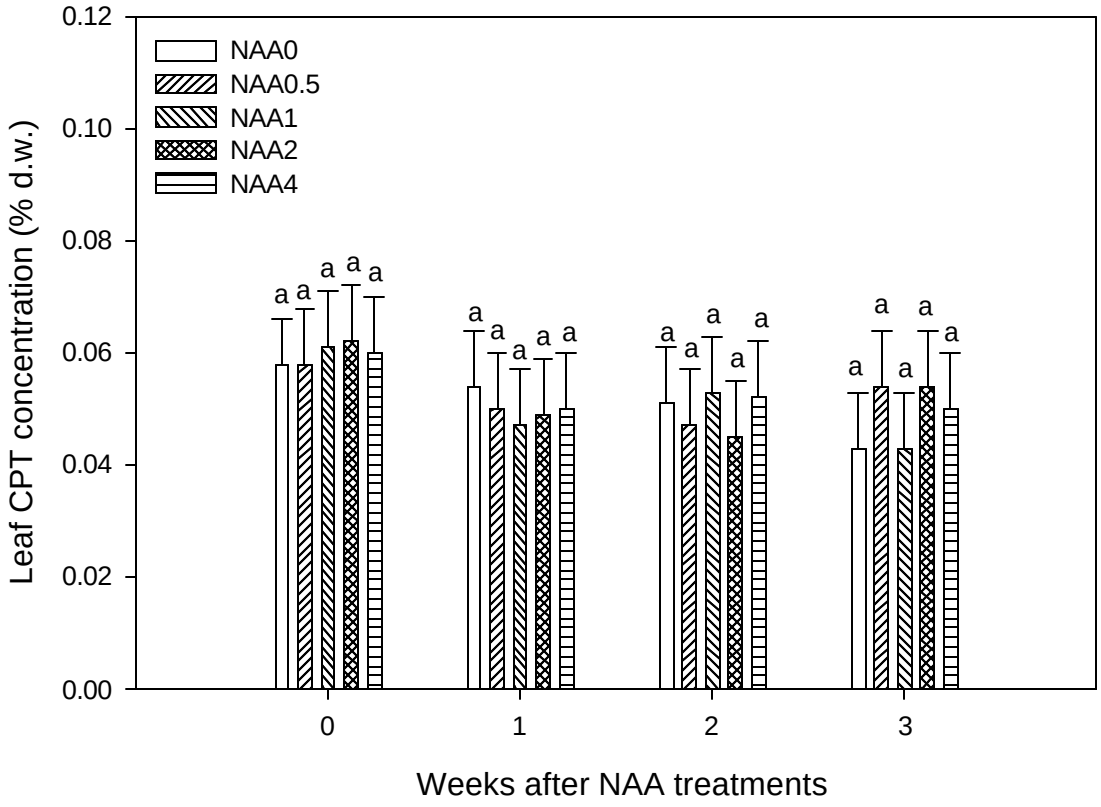

Fig. 5.7-2. Effect of NAA on leaf CPT accumulation of *C. acuminata* seedlings in a hydroponic system. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant difference of t tests among the lsmeans at $P \leq 0.05$.

Table 5.8-1. Analysis of variance of BA effect on CPT yield

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	25	2.35	0.0970
BA	3	25	1.01	0.4043

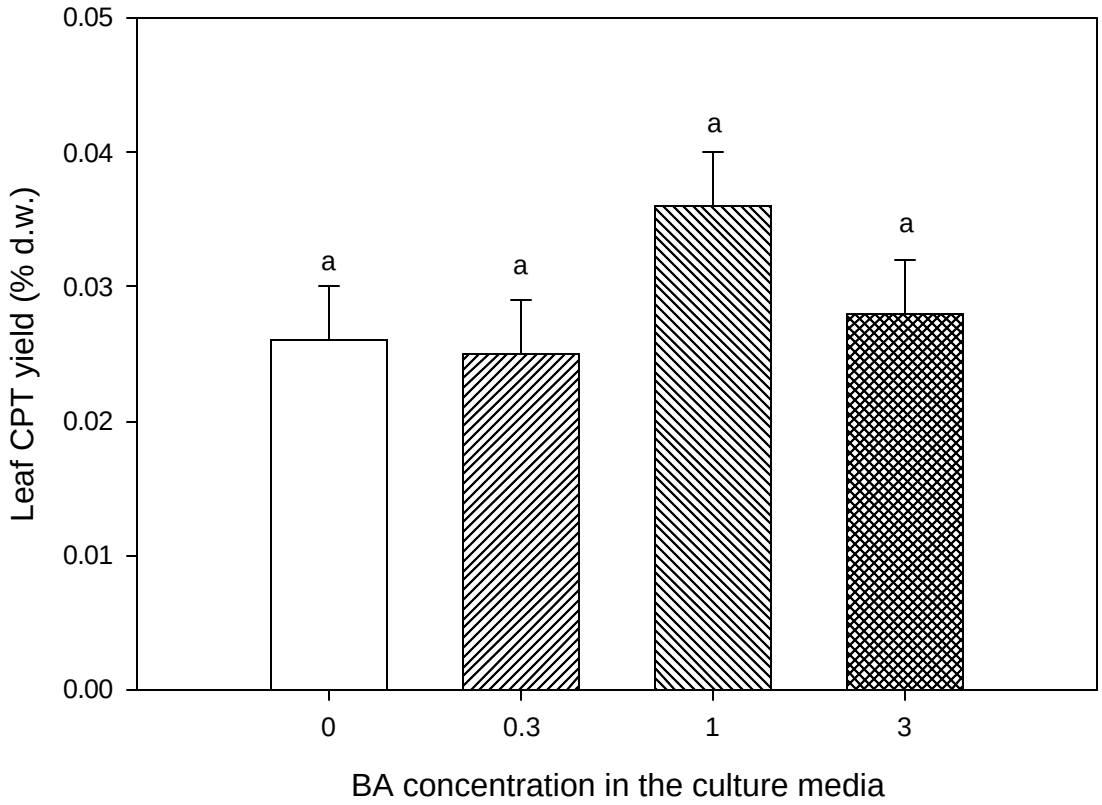

Fig. 5.8-1. Effect of BA on CPT yield of *C. acuminata* seedlings in a hydroponic culture system at week 3 of the treatments. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate the significant difference of t tests among the lsmeans at $P \leq 0.05$.

Table 5.8-2. Analysis of variance of NAA effect on CPT yield

Effect	Numerator degree freedom	Denominator degree freedom	F-value	P-value
Block	3	32	5.21	0.0048
NAA	4	32	4.71	0.0042

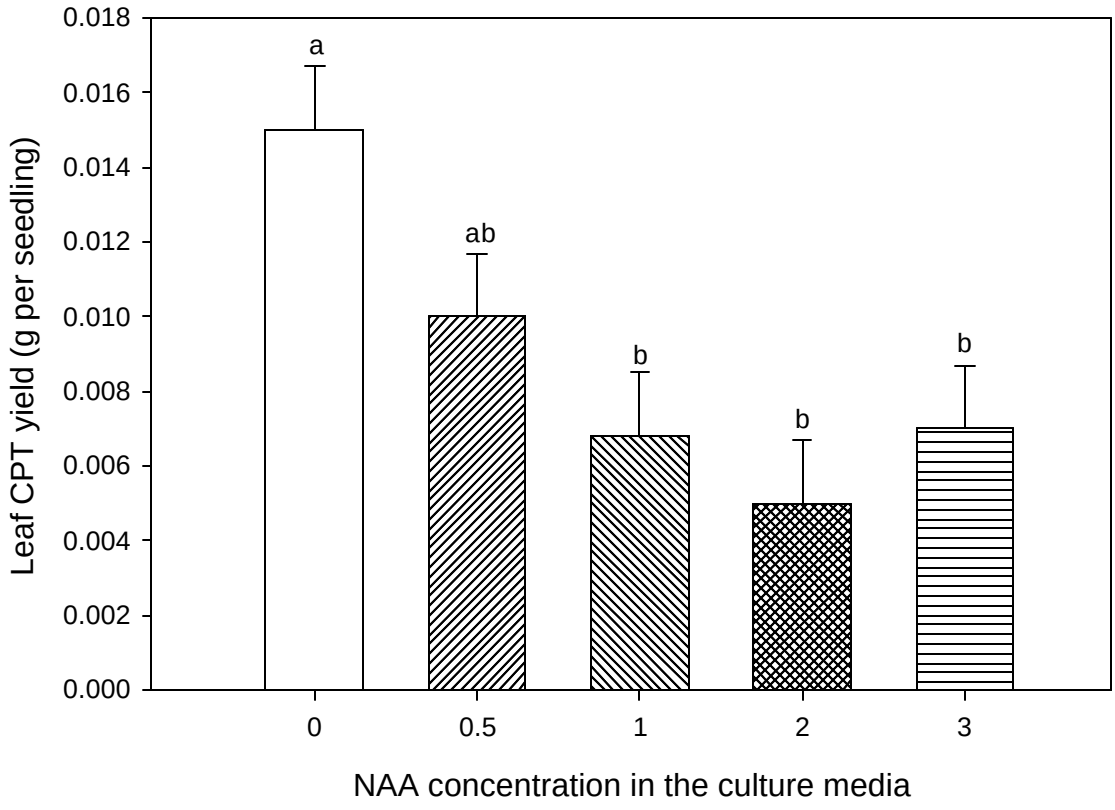

Fig. 5.8-2. Effect of NAA on CPT yield of *C. acuminata* seedlings in a hydroponic culture system at week 3 of the treatments. Vertical lines above each bar represent standard errors of lsmeans (n=8). Different letters above the bars indicate significant difference of t tests among the lsmeans at $P \leq 0.05$.

CPT Accumulation and Plant Growth, Morphology

Covariate analysis with backward variable selection for the BA study showed that RSR was significantly related to CPT accumulation, and leaf length was related slightly to CPT accumulation (Table 5.9-1). A positive relation was found between RSR and CPT accumulation, whereas a negative relation was found between leaf length and CPT accumulation.

Covariate analysis with backward variable selection for the NAA study showed that plant height was slightly related to CPT accumulation (Table 5.9-2). A negative relation was found between plant height and CPT accumulation.

Table 5.9-1. Covariate analysis with backward variable selection for BA study

Variable	Estimate	Type II SS	F-value	P-value
Intercept	0.03684	0.00127	1.47	0.2359
Leaf length	-0.01125	0.00295	3.40	0.0759
RSR	0.06734	0.01049	12.10	0.0017

Table 5.9-2. Covariate analysis with backward variable selection for NAA study

Variable	Estimate	Type II SS	F-value	P-value
Intercept	0.08568	0.02649	18.50	0.0001
Height	-0.00235	0.00414	2.89	0.0984

Discussion and Conclusions

Plant Growth and Morphology

BA and NAA applications significantly reduced plant growth. A possible reason for this might be that BA applications caused endogenous cytokinin levels in seedlings beyond their optimal levels, whereas the optimal levels might occur in the BA control seedlings. Similarly, NAA applications might cause endogenous auxin levels in seedlings beyond their optimal levels, which might occur in the NAA control seedlings.

BA and NAA both increased RSR and decreased SLW. It is possible that BA and NAA applications increased root growth in response to the abnormal hormone levels as observed in

our experiment. BA and NAA applications might also inhibit the accumulation of photosynthates in leaves and so induce smaller SLW.

CPT Accumulation and Possible Mechanism

This study showed that high BA levels significantly increased leaf CPT accumulations after a period of three weeks. It was found that the production of alkaloid in *Fagara zanthoxyloids* cell lines strongly correlated with the presence of exogenous BA, because the levels of alkaloids were 9 times lower when cells were cultured without cytokinin than in the control culture (Couillerot et al.1996). In another species, the accumulation of the indole alkaloid ajmalicine and that of a polypeptide of 28 kD were induced by 2,4-dichlorophenoxyacetic acid removal in periwinkle cell cultures and further increased by addition of zeatin to the culture medium (Carpin et al. 1997). Another cytokinin, kinetin increased total alkaloid content in both leaf and stem calli compared with the control in *Datura stramonium* in vitro culture (El Bahr et al. 1989). We hypothesized that cytokinin may serve as a signaling substance for regulating secondary metabolite accumulation. This is supported by the fact that cytokinin stimulates cell differentiation, and cell differentiation has been shown to be a prerequisite for alkaloid accumulation in many studies, e.g., the cultured tissues and organs of *Duboisia myoporoides* (Khanam et al. 2000). Some studies were done in order to identify molecular markers of the promotion action of cytokinin on indole alkaloid accumulation in a *C. roseus* cell line. For example, a group of polypeptides, the levels of which were positively controlled by cytokinin, might be implicated in the regulation of alkaloid accumulation (Ouelhazi et al. 1993). These polypeptides are candidates for a direct or indirect regulatory role in alkaloid biosynthesis in *C. roseus* cells, particularly a polypeptide of 28 kDa whose syntheses are enhanced by cytokinin (Ouelhazi et 1994).

Our study showed that NAA applications did not affect leaf CPT accumulations throughout the 3-week experiment. It is possible that auxin had no effect on CPT biosynthesis. However, in many other studies, auxins had been shown to inhibit alkaloid biosynthesis and accumulation. For example, in *C. roseus* cell cultures, auxin exerted a dramatic inhibition on alkaloid terpenoid precursor availability, and during one subculture, the auxin depletion greatly enhanced the ajmalicine content (Gantet et al. 1997). Auxins have also been shown to down-regulate gene expressions in alkaloid biosynthesis. In *C. roseus* cell suspension cultures the *sss* gene (for strictosidine synthase) and *tdc* gene (for tryptophan decarboxylase) were rapidly down-regulated by auxin, but both genes were strongly induced by fungal elicitors (Pasquali et al. 1992). Omission of NAA from the *C. roseus* cell suspension growth medium resulted in the accumulation of *tdc* mRNA. The addition of NAA, indole acetic acid (IAA), or 2,4-dichlorophenoxy acetic acid (2,4-D) rapidly reduced the enhanced *tdc* transcript level. Thus one of the mechanisms which controls the activity of terpenoid indole alkaloid biosynthesis in *C. roseus* cell cultures was the negative regulation by auxin of the genes involved in the first committed step (Goddijn et al. 1992).

CPT Yield

Final leaf CPT yields were similar across the BA applications, because final CPT yield in leaves depended on two factors: plant biomass and CPT accumulation. Although high BA levels produced higher CPT accumulations, they had slightly lower plant biomasses and in this case they produced similar CPT yields as low BA levels.

Final leaf CPT yield decreased with increasing NAA accumulations and the highest CPT yield was in the NAA control. Leaf CPT accumulations were similar across all NAA applications, but plant biomasses decreased with increasing NAA accumulations, so CPT yields

decreased with increasing NAA levels. On the other hand, the NAA control produced the highest CPT yield, because it had similar CPT accumulation and similar plant weight as the low NAA levels but it produced the highest leaf number, leaf length, and more shoots.

CPT Accumulation and Plant Growth, Morphology

In the BA study, a significant positive relation was found between RSR and CPT accumulation. A possible reason might be that increased plant RSR or root growth was a response to environmental stress, such as the toxic levels of cytokinin, and CPT accumulation usually increased as a consequence of environmental stress, so RSR and CPT accumulation were positively related. A slight negative relation was found between leaf length and CPT accumulation. A possible explanation was that there was a trade-off between plant growth and CPT accumulation. Under stress conditions, CPT accumulation was invoked and plant growth was inhibited, thus producing small leaf length.

In the NAA study, a slight negative relation was found between plant height and CPT accumulation. An explanation was that a trade-off between plant growth and CPT accumulation might exist. For example, improved alkaloid production was found to be related to a slower overall growth rate in suspended *Papaver somniferum* culture (Siah and Doran 1991).

In conclusion, exogenous BA and NAA applications inhibited plant height and leaf number of *C. acuminata* in a hydroponic culture system, and NAA applications also inhibited plant weight and leaf length. Exogenous BA and NAA applications both increased RSR, and NAA applications decreased SLW. BA applications significantly increased CPT accumulation, whereas NAA applications had no effect on CPT accumulation. Conversely, BA applications had no effect on CPT yield, and NAA application decreased CPT yield.

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GENERAL CONCLUSIONS

Effects of several abiotic and biotic factors on plant growth, morphology, CPT accumulation, and CPT yield in *C. acuminata* were explored. In addition, effects of plant hormones on *C. acuminata* were also studied. In these studies, *C. acuminata* seedlings in a hydroponic culture system with commercial woody plant media and fluorescent lights and at a room temperature (22-26°C) were used, and the following results were found.

N deficiency significantly decreased plant height, weight, leaf number, leaf length, and leaf chlorophyll accumulation in comparison with N supplementation from 7.5 to 30 mM, but increased root to shoot ratio (RSR), specific leaf weight (SLW), and CPT accumulations in leaves and roots, and did not increase CPT yield.

NaCl addition significantly reduced plant height, weight, leaf number, and leaf length, but increased SLW and CPT accumulations in leaves and roots, and decreased CPT yield.

UV light short-term exposure had no effect on plant height, weight, leaf number, leaf length, RSR, and SLW, but increased leaf CPT accumulation and CPT yield after a period of three weeks.

Acetylsalicylic acid (ASA) addition significantly reduced plant height, weight, and leaf length, but increased SLW and leaf CPT accumulation, and decreased CPT yield.

BA application significantly decreased plant height, but increased RSR and leaf CPT accumulation, and had no effect on CPT yield. NAA application significantly decreased plant height, weight, leaf number, leaf length, SLW, and CPT yield, but increased RSR, and had no effect on leaf CPT accumulation.

In conclusion, abiotic and biotic stresses reduced plant growth and affected plant morphology of *C. acuminata* in a hydroponic culture system. The abiotic stresses, such as N deficiency, high NaCl accumulation, and UV light, and the biotic stress, such as ASA at high accumulation all increased the plant secondary metabolite CPT accumulation, but did not increase final CPT yield (expect for UV). The plant hormones, cytokinin up-regulated CPT accumulation but had no effect on CPT yield, whereas auxin did not regulate CPT accumulation but decreased CPT yield. A negative relationship between plant growth and CPT accumulation existed under the treatments with N, NaCl, or ASA.

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