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Effects of Benzyladenine and Naphthalene Acetic Acid on Growth and Camptothecin Accumulation in *Camptotheca Acuminata* Seedlings

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Abstract

The effects of the cytokinin benzyladenine (BA) and the auxin naphthalene acetic acid (NAA) on *Camptotheca acuminata* Decaisne growth and camptothecin (CPT) accumulation (leaf CPT concentration and total leaf CPT yield) were studied in a hydroponic culture system for three weeks. Increasing BA concentrations from 0 to 3 mg l^{-1} in growth medium decreased plant height, stem weight, and leaf weight but increased root weight. High BA levels (1 and 3 mg l^{-1}) increased leaf CPT concentration (% of dry weight), whereas BA applications had no effect on total leaf CPT yield, the product of leaf CPT concentration and total leaf dry weight per seedling. There was a positive correlation between root weight and leaf CPT concentration under BA treatments. NAA supplementations (from 0.5 to 4 mg l^{-1}) to growth medium reduced plant height, leaf number, leaf length, specific leaf weight, plant weight, stem weight, and leaf weight compared with the NAA control. Meanwhile, there were no differences in plant height, leaf length, and specific leaf weight among the NAA supplementations. NAA applications had no effect on leaf CPT concentration and NAA applications decreased total leaf CPT yield. There were negative correlations between leaf number and leaf CPT concentration, leaf length and leaf CPT concentration under NAA treatments. Our results suggest that BA applications from 0.3 to 3 mg l^{-1} are not helpful for achieving high total leaf CPT yield and NAA applications from 0.5 to 4 mg l^{-1} decrease total leaf CPT yield.

Key words: Alkaloid; Anti-cancer plant; Auxin; BA; *Camptotheca acuminata* Decaisne (family Nyssaceae); Camptothecin; Cytokinin; NAA

Introduction

Camptotheca acuminata Decaisne (family Nyssaceae) is a deciduous tree species indigenous to southern China. Like many terpenoid indole alkaloids, produced by *Cathranthus roseus* and used for the treatment of various tumors (Carpin and others 1997), camptothecin (CPT), produced by *C. acuminata*,

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exhibits anti-cancer activity. This activity was first identified by Wall and others (1966) and is due to the substance's ability to inhibit DNA topoisomerase I (Kjeldsen and others 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. Sources of these two compounds rely on the extraction of CPT from raw plant material and chemical modification (methoxylation and/or hydroxylation) of extracted CPT in laboratories to reduce its toxicity and to increase its activity. Unlike *Mappia foetida,* another slow-growing species producing CPT, *C. acuminata* is a fast-growing species. Hence, production of CPT from *C. acuminata* plant material remains an important source of these therapeutic compounds.

Because of the promising clinical uses of CPT, it is important to investigate factors affecting CPT yield and to extend our understanding of CPT accumulation. Here, we study CPT accumulation in two aspects: CPT concentration (in leaf) and (total leaf) CPT yield (the product of leaf CPT concentration and total leaf dry weight per seedling). The basis for comparison of (leaf) CPT concentration is % of dry weight and the basis for comparison of (total leaf) CPT yield is (total leaf) CPT content (g) per seedling. CPT is a terpenoid indole alkaloid. As a secondary metabolite, it is hypothesized to defend the plant against stresses (Liu and Adams 1996; Liu and others 1997, 1998). CPT accumulates in leaves, stems and roots of *C. acuminata* seedlings, and leaves have relatively higher CPT concentrations than stems and roots (Liu and others 1997, 1998; our unpublished data). Therefore, leaves are mainly used in repeated and non-destructive harvesting for CPT (Lopez-Meyer and others 1994). However, the exact site of CPT biosynthesis is still unknown, and there is no report on translocation of CPT, a water-insoluble compound, within *C. acuminata*.

Many factors affect the accumulation of CPT in *C. acuminata.* CPT concentrations varied significantly with leaf age and tree age; high CPT concentrations were found in young leaves and young trees (Liu and others 1998). Shading of whole seedlings increased CPT concentration in leaves and decreased CPT concentration in roots (Liu and others 1997). In a greenhouse study, drought increased the CPT concentration in leaves (Liu 2000). A moderate mixed fertilizer added to *C. acuminata* seedlings in the field either decreased or had no effect on CPT concentration (Liu and others 1999). Methyl jasmonate (MeJa) and yeast extract treatments on leaf discs punched from *C. acuminata* seedlings promoted the expression of tryptophan decarboxylase mRNA, a key enzyme involved in CPT biosynthesis (Lopez-Meyer and Nessler 1997). Similarly, MeJa and yeast extract treatments on *C. acuminata* cell suspension cultures increased CPT accumulation (Song and Byun 1998).

This study explored the effects of plant hormones, cytokinin and auxin on CPT accumulation in *C. acuminata.* Cytokinins, such as benzyladenine (BA), zeatin, and kinetin have important physiological effects on plant growth. For example, they promote shoot formation and lateral bud expansion, and delay leaf senescence through their roles in inducing cell division and cell differentiation (Taiz and Zeiger 1998). Cytokinins have also been shown to be involved in plant secondary metabolism. For example, cytokinins were found to stimulate alkaloid biosynthesis in cell lines of *C. roseus* (Decendit and others 1992). We hypothesize that cytokinins also affect CPT accumulation in *C. acuminata*.

Auxins, such as naphthalene acetic acid (NAA) and indole acetic acid (IAA) also have important physiological effects on plant growth. They promote apical dominance, lateral and adventitious root formation, and stem and leaf elongation by their roles in inducing cell elongation (Taiz and Zeiger 1998). They also play roles in regulating plant secondary metabolism. For example, in transformed root cultures of *Hyoscyamus muticus,* the growth of roots was not affected by NAA, but the accumulation of alkaloid was doubled compared with roots growing in the absence of auxin, that is, alkaloid concentration had been increased by exogenous auxin without influencing growth (Vanhala and others 1998). Conversely, in *C. roseus* cell suspensions, omission of 2,4-dichlorophenoxy acetic acid (2,4-D) from the medium resulted in an increased alkaloid concentration (Avry and others 1994). We hypothesize that auxins affect CPT accumulation in *C. acuminata*.

In our study, BA, a classic cytokinin, and NAA, a classic auxin, were used. To study the effects of BA and NAA on (total leaf) CPT yield, we collected data on plant growth and (leaf) CPT concentration, all related to CPT yield. On one hand, the effects of these factors on plant growth were considered because more plant growth could provide more plant biomass, and relatively more leaves could bring more useful material to harvest. On the other hand, CPT concentrations in leaves was of concern because higher CPT concentrations in leaves could provide an efficient way to increase CPT yield. Finally, understanding the relationship between plant growth and (leaf) CPT concentration could

extend our knowledge of CPT accumulation and plant secondary metabolism.

This study was done in a *C. acuminata* hydroponic culture system, which provided us with well-controlled nutrients, light, temperature, humidity, and precisely controlled treatments, such as BA and NAA concentrations, in contrast to various field conditions. The specific aims of this study were to evaluate the effects of BA and NAA on *C. acuminata* growth, (leaf) CPT concentration, and (total leaf) CPT yield and to define the relationship between plant growth and (leaf) CPT concentration.

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 containing 6 l of half-strength woody plant medium (Sigma Chemical, St. Louis, MO) solution supplemented with 2 mg l^{-1} (9.8 μM) indole butyric acid and adjusted to pH 6.5 and enclosed in a mini-chamber. After 2–4 weeks, the rooted seedlings of similar height (about 10 cm) and similar leaf number (about six leaves) were selected and placed in plastic containers containing 100 ml of half-strength woody plant medium solution in chambers. Seedlings were allowed to acclimate in chambers for one week prior to experimentation.

Two separate experiments were conducted to test for the effects of BA and NAA on plant growth and CPT accumulation. In the BA study, 32 seedlings were chosen and divided equally into four groups, with each group assigned to one of four chambers (blocks). In each chamber, seedlings were randomly assigned to a BA level of 0, 0.3, 1, or 3 mg l^{-1} (0, 1.32, 4.4, or 13.2 μM). BA application was done as follows: 3.2 l half-strength woody plant medium solution was prepared and divided equally into four flasks. BA stock solution was 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 new seedling container and a seedling was then placed into the container. Each BA treatment was replicated twice within each of four chambers. Seedlings were grown under fluorescent lights providing 40 μmol m^{-2} s^{-1} light intensity and a 16 h photoperiod at room temperature (22–26°C). The NAA study was performed in a similar manner, 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 (cm), weight (fresh, g), leaf number, and blade length (cm) of the third leaf from shoot apex (leaves longer than 1.5 cm were considered to be the first leaves) were measured at the beginning (week 0) and weeks 1, 2, and 3 of the treatments. Plant weight referred to the fresh weight of the entire plant including roots. Fresh weights were used because total plant dry weights could not be obtained at weeks 0, 1, and 2. Total plant fresh weights were measured at weeks 0, 1, 2, and 3, so plant weights could be compared. Following these measurements, two circular discs (5 mm in diameter) were punched (in the middle of the blade and beside the main vein to reduce the damage to seedlings and to minimize the possible CPT variation in leaf size and disc location on leaves) from each of the fifth and sixth leaves on each seedling. In our previous study we 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 and others 1998). Punching two small discs (5 mm in diameter) from each of the fifth and sixth leaves was shown not to affect leaf CPT concentrations in our preliminary experiment (unpublished data). The collected leaf discs were immediately put into a 1.5-ml pre-weighed Eppendorf tube, frozen in liquid N_2, 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, and weighed again after oven-drying at 70°C for 72 h at the end of the experiment. Root weight, stem weight, and leaf weight were obtained from the fresh weights of roots, stems, and leaves, which could be compared to the total plant fresh weight. Specific leaf weight (SLW, dry leaf weight per unit leaf area, mg cm^{-2}) was derived from the punched leaf discs.

The freeze-dried leaf samples were ground with a pestle in the Eppendorf tubes with 50 μl absolute methanol added first to eliminate static electricity. After grinding, an additional 950 μl of methanol was added to the tube. Methanol was found to be an optimal extraction solvent for CPT in our previous studies, showing 6- and 3-fold more power than dichloromethane and acetone, respectively, in extracting CPT from the leaf samples. The samples in locked tubes were extracted in methanol for CPT on a rotator for about 16 h 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 by air blowing under a

Table 1. *P*-values from Repeated Measures Analyses and Analyses of Variance for the Effects of BA or NAA on Measures of Plant Growth and CPT Accumulation (leaf CPT concentration and total leaf CPT yield)

	BA Study			NAA Study		
Variable	BA	Time	BA*Time	NAA	Time	BA*Time
Plant height	0.21	<0.001	<0.001	<0.001	<0.001	<0.001
Leaf number	0.19	<0.001	0.16	<0.001	<0.001	<0.001
Leaf length	0.44	<0.001	0.11	0.16	<0.001	0.016
SLW	0.083	0.49	0.37	0.024	<0.001	0.034
Plant weight	0.18	<0.001	0.78	0.002	<0.001	<0.001
Root weight	0.013	—	—	<0.001	—	—
Stem weight	<0.001	—	—	<0.001	—	—
Leaf weight	<0.001	—	—	<0.001	—	—
Leaf CPT concentration	0.61	0.017	0.049	0.70	0.80	0.094
Total leaf CPT yield	0.40	—	—	<0.001	—	—

hood and reconstituted with 100 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 and others 1998). The analytical method yielded an average of 96% recovery of standard CPT (99% purity). Leaf CPT concentration was expressed as a percentage (%) of the dry weight of measured leaf material. Final CPT yield (actually total leaf CPT yield) was defined as total leaf CPT content (g) per seedling, that is, the product of leaf CPT concentration and total leaf dry weight per seedling at the end of experiment.

Repeated measures analyses were performed for the effects of BA and time on plant height, leaf number, leaf length, SLW, plant weight, and leaf CPT concentration using the SAS software. Analyses of variance were performed for the effects of BA on root weight, stem weight, leaf weight, and total leaf CPT yield. Least square means (lsmeans) were obtained and compared by Student's *t* test. Pearson correlation coefficients between leaf CPT concentration and all measures of growth were evaluated at the same time point (week 3) to identify which variables were significantly related to leaf CPT concentration. Similar analyses were performed for the NAA study. All tests were regarded as significant at $P \leq 0.05$.

Results

BA Study

The effect of BA on the height of *C. acuminata* seedling depended on time ($P < 0.001$) (Table 1). BA applications ranging from 0 to 3 mg l^{-1} had no effect on plant height at weeks 0, 1, and 2, whereas plant height increment declined significantly with increasing BA at week 3 (Figure 1A). No significant interaction between BA and time on leaf number was found ($P = 0.16$) and BA had no effect on leaf number ($P = 0.19$; Table 1). Leaf numbers were similar among all BA treatments at weeks 0, 1, 2, and 3 (Figure 1B). There appeared to be no significant interaction between BA and time on leaf length measured on the third leaf ($P = 0.11$), and there was no significant effect of BA on leaf length ($P = 0.44$, Table 1). Leaf length did not differ across all BA treatments at weeks 0, 1, 2, and 3 (Figure 1C). There was no significant interaction between BA and time on SLW measured on the fifth and sixth leaves ($P = 0.37$) and there was no significant effect of BA on SLW ($P = 0.083$, Table 1). SLW did not change across all BA treatments at weeks 0, 1, 2, and 3 (Figure 1D). There was no significant interaction between BA and time on plant fresh weight ($P = 0.78$) and BA applications in the solution had no effect on plant weight ($P = 0.18$, Table 1, Figure 2A). BA applications had a significant effect on root fresh weight at week 3 ($P = 0.013$) (Table 1) and increasing BA concentration increased root weight (Figure 3A). BA applications had a significant effect on stem fresh weight at week 3 ($P < 0.001$, Table 1) and increasing BA concentration decreased stem weight (Figure 3B). BA applications significantly affected total leaf fresh weight ($P < 0.001$, Table 1) and increasing BA concentration led to decreased total leaf weight at week 3 of the treatments (Figure 3C).

There was a significant interaction between BA and time on leaf CPT concentration measured from the fifth and sixth leaves ($P = 0.049$, Table 1). Prior

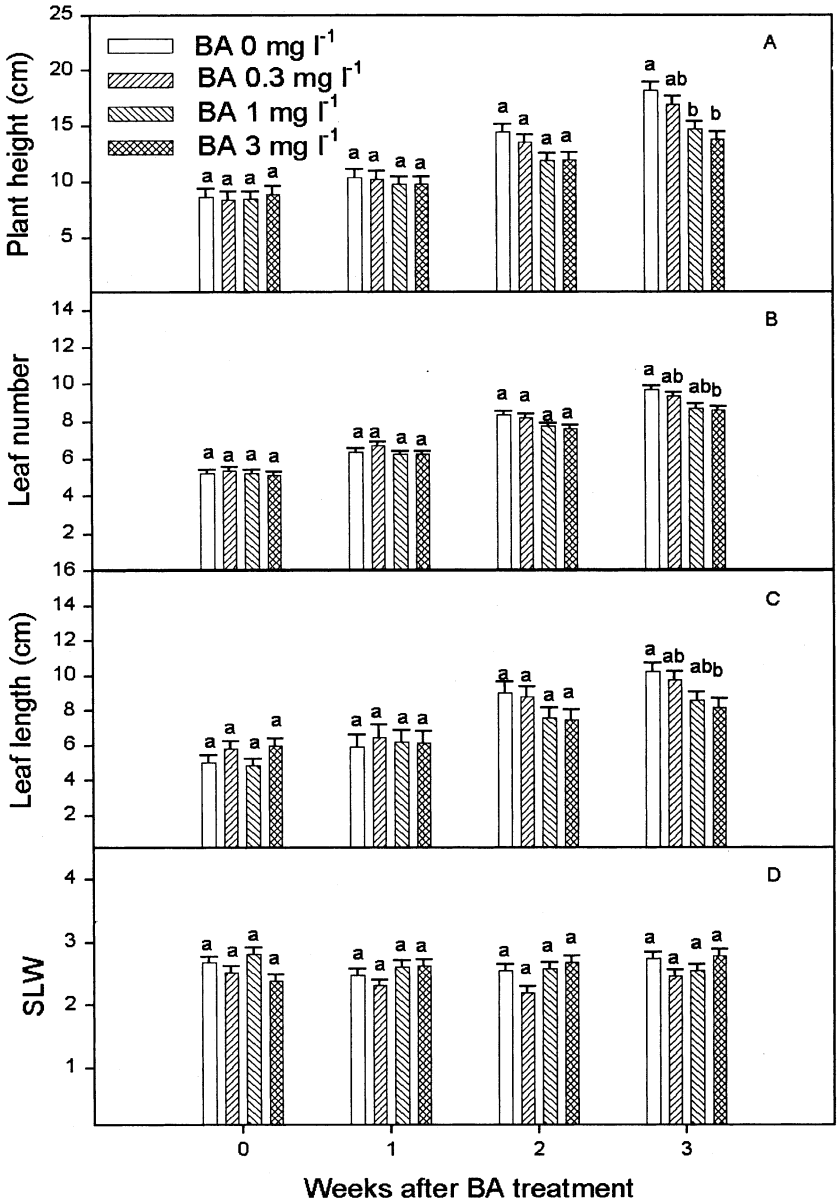

Figure 1. Effects of BA on plant height (**A**), leaf number (**B**), blade length of the third leaf from shoot apex (**C**), and SLW (mg dry weight/ cm^{-2}) on the fifth and sixth leaves (**D**) of *C. acuminata* seedlings. Vertical lines and different letters above the bars indicate standard errors (SE) and significant differences among lsmeans, respectively.

to BA treatments, there were no differences in leaf CPT concentrations among the plants, indicating homogeneous experimental materials (Figure 2B). There were no differences in leaf CPT concentrations one and two weeks after BA treatments. However, leaf CPT concentrations were significantly higher in seedlings treated with high levels of BA (1 and 3 mg l^{-1}) three weeks after treatments. BA applications had no effect on the final total leaf CPT yield, the product of leaf CPT concentration and total leaf dry weight per seedling ($P = 0.40$, Table 1, Figure 3D). Pearson correlation coefficients between leaf CPT concentration and all measures of growth are given for the BA study (Table 2). Root weight was significantly and positively related to leaf CPT concentration ($r = 0.37$ and $P = 0.035$). On the other hand, none of the other measures of growth was significantly related to leaf CPT concentration.

NAA Study

The effect of NAA on plant height depended on time ($P < 0.001$, Table 1). NAA applications ranging from 0 to 4 mg l^{-1} produced no differences in plant height at weeks 0 and 1, whereas plant height increment declined significantly with NAA supplementations at weeks 2 and 3 compared with the NAA control. There were no differences in plant height among the various NAA concentrations (Figure 4A). The effect of NAA applications on leaf number depended

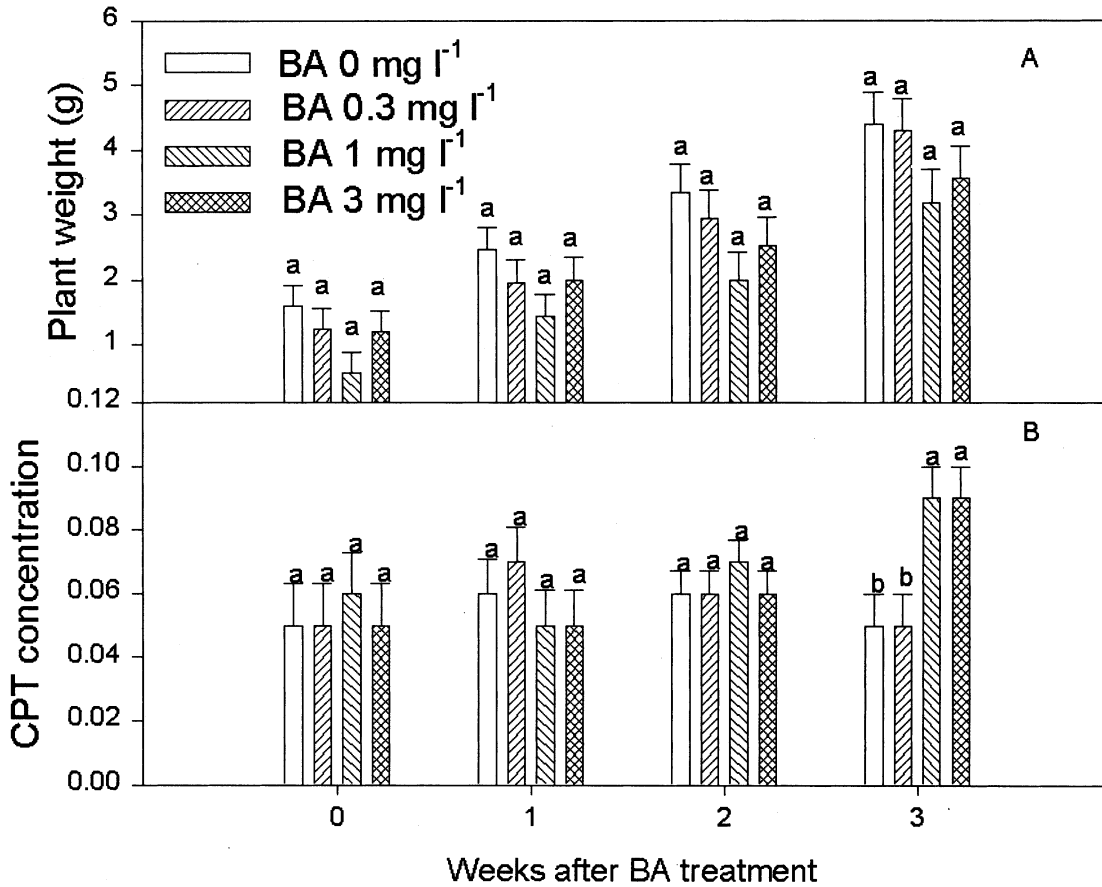

Figure 2. Effects of BA on plant weight (fresh) (**A**) and leaf CPT concentration (% of the dry weight of the material) measured from the fifth and sixth leaves (**B**) of *C. acuminata* seedlings. Vertical lines and different letters above the bars indicate SE and significant differences among lsmeans, respectively.

on time ($P < 0.001$, Table 1). There were no differences in leaf number at weeks 0 and 1, but leaf number increments declined significantly with increasing NAA levels (except for the highest level) at weeks 2 and 3. At the highest NAA level, leaf number increments were not significantly reduced compared to the control (Figure 4B). The effect of NAA on the length of the third leaf depended on time ($P = 0.016$, Table 1). NAA applications from 0 to 4 mg l^{-1} produced no differences in leaf lengths at weeks 0 and 1, but showed significant differences in leaf length at weeks 2 and 3 (Figure 4C). NAA supplementations from 0.5 to 4 mg l^{-1} significantly reduced leaf length compared with the control, whereas there were no differences in leaf length among treatments at levels of 0.5 to 4 mg l^{-1} NAA. There was a significant interaction between NAA and time on SLW measured on the fifth and sixth leaves ($P = 0.034$, Table 1). There were no differences in SLW among NAA treatments at weeks 0, 1, and 2, but NAA supplementations significantly reduced SLW compared with the NAA control at week 3 (Figure 4D). NAA supplementation levels from 0.5 to 4 mg l^{-1} produced similar SLW values at week 3. There was a significant interaction between NAA and time on plant fresh weight ($P < 0.001$, Table 1). There were no differences in plant weight at weeks 0 and 1, but plant weight increment decreased significantly with increasing NAA concentrations from 0 to 4 mg l^{-1} at weeks 2 and 3 (Figure 5A). NAA applications had a significant effect on root fresh weight at week 3 ($P < 0.001$, Table 1) and NAA at 0.5 mg l^{-1} produced the highest root weight (Figure 6A). NAA applications had a significant effect on stem fresh weight at week 3 ($P < 0.001$, Table 1) and increasing the NAA concentration decreased stem weight (Figure 6B). NAA applications significantly affected the total leaf fresh weight ($P < 0.001$, Table 1) and increasing the NAA concentration decreased total leaf weight at week 3 (Figure 6C, Panel 3).

There was no significant interaction between NAA and time on leaf CPT concentrations measured

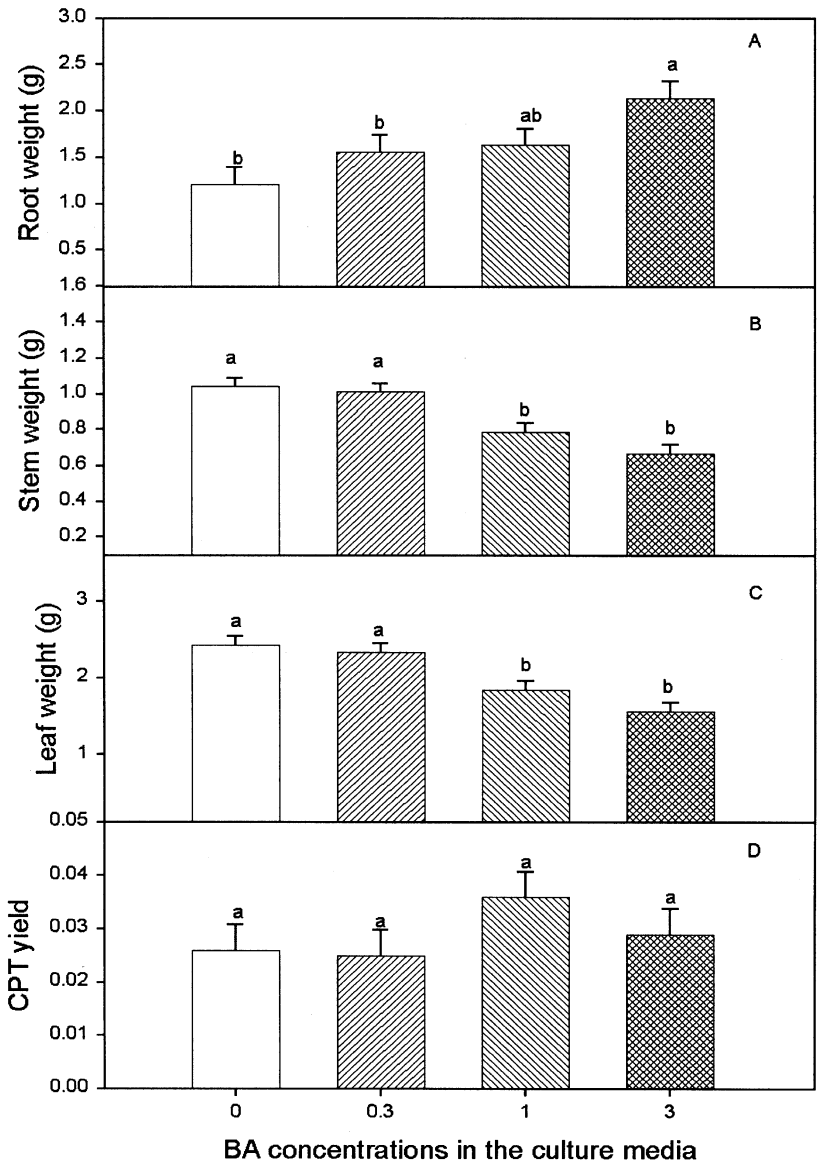

Figure 3. Effects of BA on the fresh weights of root (**A**), stem (**B**), total leaf (**C**), and total leaf CPT yield (g) (**D**) of *C. acuminata* at week 3. Vertical lines and different letters above the bars indicate SE and significant differences among lsmeans, respectively.

Table 2. Correlations between leaf CPT concentration and measures of plant growth

	BA Study		NAA Study	
Variable	Coefficients	*P*-values	Coefficients	*P*-values
Plant height	−0.14	0.44	−0.28	0.098
Leaf number	0.01	0.97	−0.34	0.043
Leaf length	−0.22	0.23	−0.47	0.004
SLW	−0.04	0.82	−0.04	0.83
Plant weight	−0.02	0.89	−0.16	0.33
Root weight	0.37	0.035	−0.17	0.31
Stem weight	−0.28	0.11	−0.26	0.10
Leaf weight	−0.28	0.12	−0.26	0.11

from the fifth and sixth leaves ($P = 0.094$) and there was no significant effect of NAA on leaf CPT concentration ($P = 0.70$, Table 1). Prior to the NAA treatments, there were no differences in leaf CPT concentrations among the treatments, and NAA applications did not change leaf CPT concentrations throughout the experiment (Figure 5B). On the other hand, increasing NAA concentrations significantly decreased the final total leaf CPT yield, the product of leaf CPT concentration and total leaf dry weight per seedling, relative to the NAA control ($P < 0.001$, Table 1, Figure 6D). Pearson correlation coefficients between leaf CPT concentration and all measures of growth for the NAA study are given in

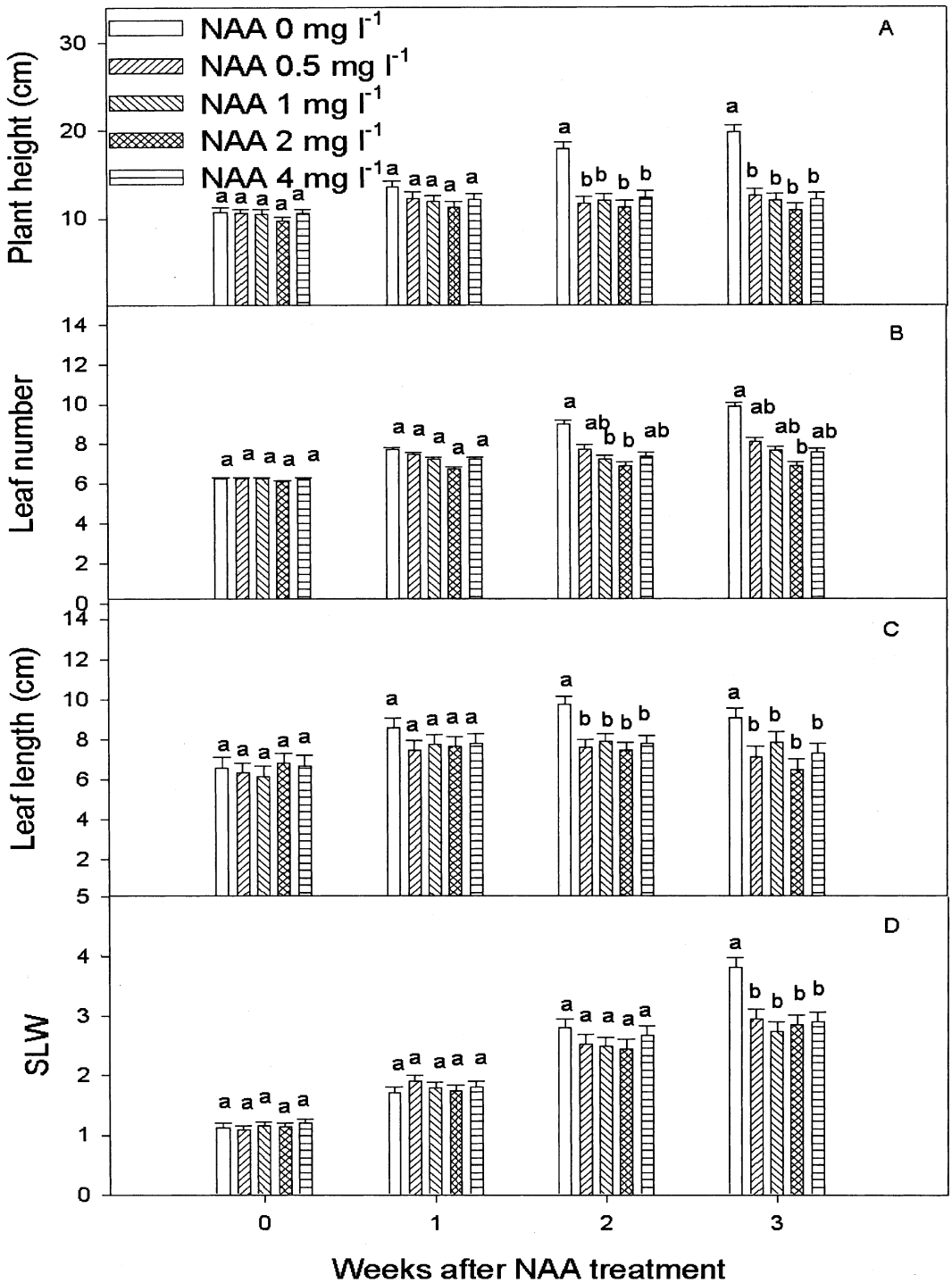

Figure 4. Effects of NAA on plant height (**A**), leaf number (**B**), blade length of the third leaf from shoot apex (**C**), and SLW (mg dry weight/cm^{-2}) on the fifth and sixth leaves (**D**) of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines and different letters above the bars indicate SE and significant differences among lsmeans, respectively.

Table 2. Leaf number and leaf length were significantly related to leaf CPT concentration ($r = -0.34$ and $P = 0.043$, $r = -0.47$ and $P = 0.004$, respectively) in a negative manner.

Discussion

In this study, BA applications reduced the height, stem weight, and leaf weight of *C. acuminata* seed-

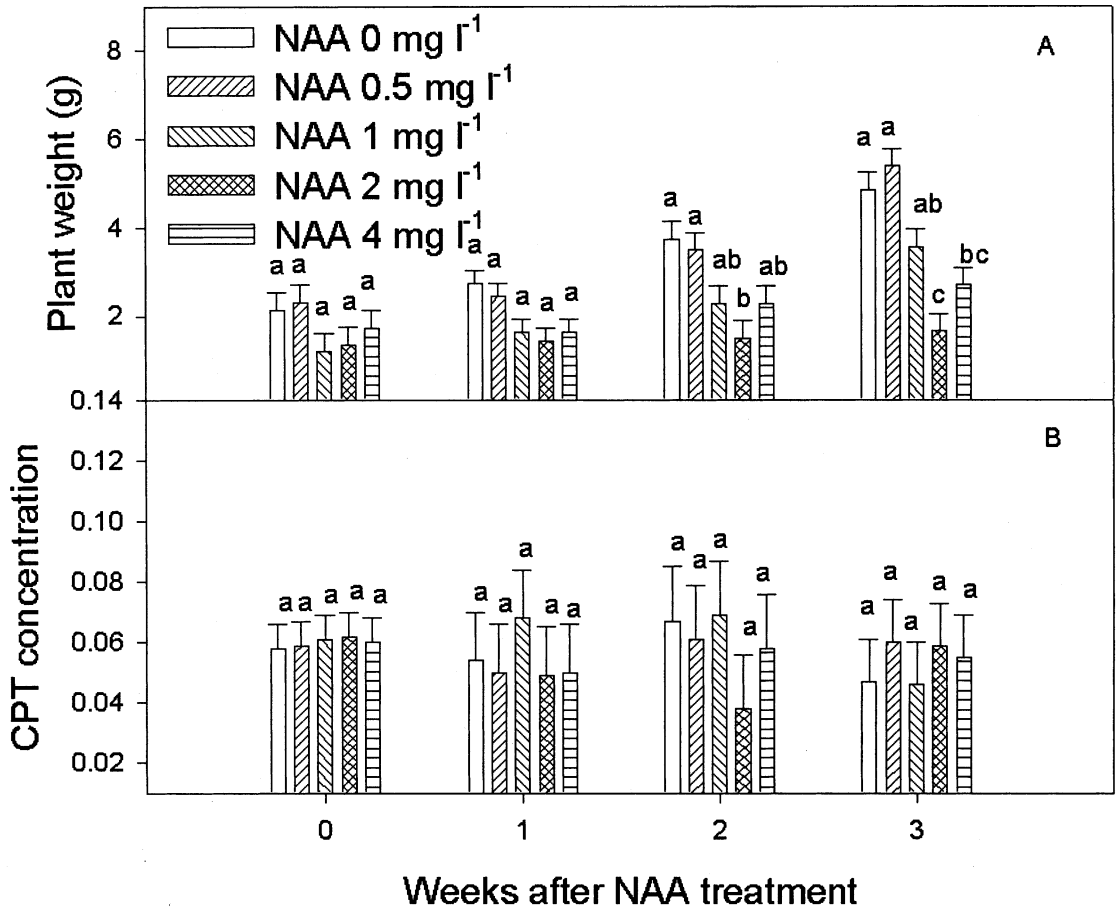

Figure 5. Effects of NAA on plant weight (fresh) (**A**) and leaf CPT concentration (% of the dry weight of the material) measured from the fifth and sixth leaves (**B**) of *C. acuminata* seedlings in a hydroponic culture system. Vertical lines and small letters above the bars indicate SE and significant differences among lsmeans, respectively.

lings at week 3. It is possible that BA applications increased cytokinin levels in seedlings beyond their optimal levels seen in the controls. BA applications increased root weight of *C. acuminata* at week 3 and produced thick roots, as observed in our experiment. These results suggest that BA applications were affecting seedlings and promoted cell differentiation in roots.

Our studies showed that high BA levels (1 and 3 mg l^{-1}) significantly increased leaf CPT concentration. The production of alkaloids in *Fagara zanthoxyloids* cell lines was strongly correlated with the presence of exogenous BA, where the levels of alkaloids were nine times lower when cells were cultured without cytokinin (Couillerot and others 1996). In periwinkle cell cultures, the accumulation of the indole alkaloid ajmalicine was increased by addition of zeatin to the medium (Carpin and others 1997). Another cytokinin, kinetin, increased the total alkaloid content in both leaf- and stem-calli compared with the control in *Datura stramonium* in vitro culture (El Bahr and others 1989). Cytokinins might serve as a signaling substance for regulating secondary metabolite accumulation. This hypothesis is supported by the fact that cytokinins stimulate cell differentiation, and cell differentiation is a prerequisite for alkaloid accumulation in many species, for example, *Duboisia myoporoides* tissue and organ cultures (Khanam and others 2000). Furthermore, in studies done to identify the molecular markers of the promoting action of cytokinin on indole alkaloid accumulation in a *C. roseus* cell line a group of polypeptides, particularly a 28 kDa polypeptide, the levels of which were positively controlled by cytokinin were thought to regulate alkaloid biosynthesis (Ouelhazi and others 1993; Ouelhazi and others 1994).

Because leaves are mainly used in repeated and non-destructive harvesting for CPT, the final CPT yield was defined as total leaf CPT yield, that is, the product of leaf CPT concentration and total leaf dry weight per seedling. The final total leaf CPT yields

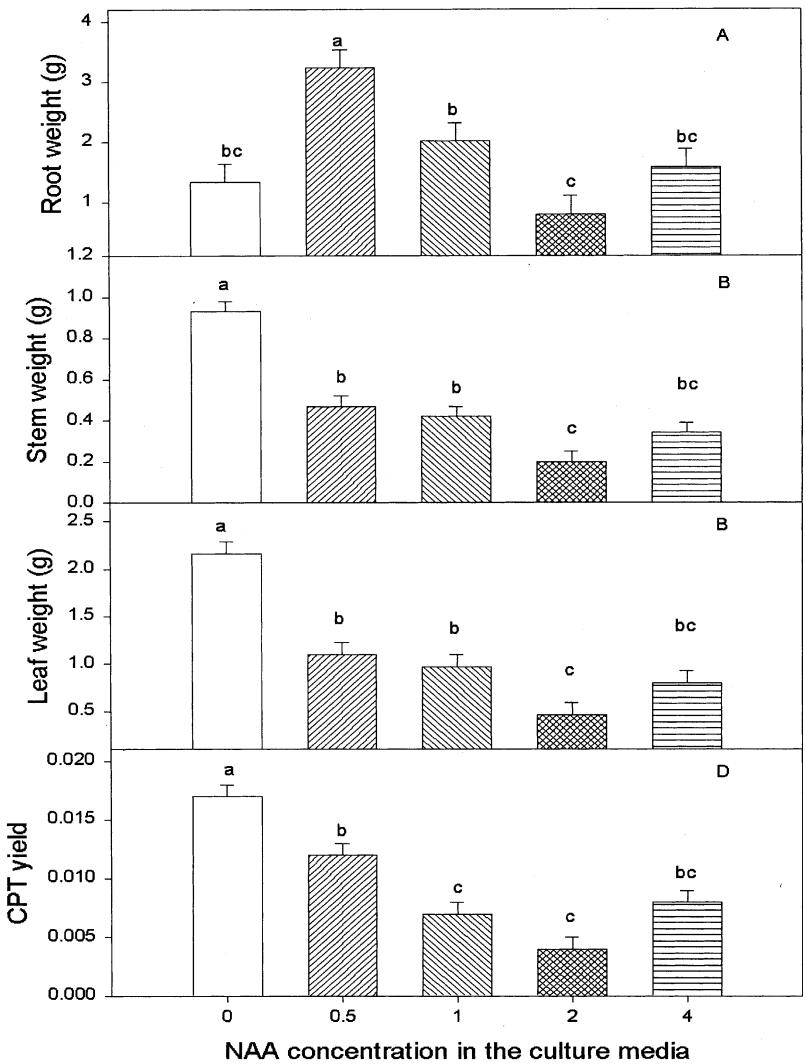

Figure 6. Effects of NAA on the fresh weights of root (**A**), stem (**B**), total leaf (**C**, Panel 3), and total leaf CPT yield (g) (**D**) of *C. acuminata* at week 3 in a hydroponic culture system. Vertical lines and small letters above the bars indicate SE and significant differences among lsmeans, respectively.

were similar across BA treatments because the final total leaf CPT yield depended on two factors: total leaf biomass and leaf CPT concentration. Although high BA levels produced higher CPT concentrations, lower total leaf biomass also resulted. Thus, high BA levels produced CPT yields similar to low BA levels and control. Our results suggest BA applications from 0.3 to 3 mg l^{-1} do not improve total leaf CPT yield. In the BA study, a significant positive relationship was found between root weight and CPT concentration in the leaves. A possible reason for this is that BA was affecting both root and leaf parts by promoting cell differentiation. On one hand, enhanced cell differentiation by BA in roots formed thick roots (as observed in our study) and increased root weight. On the other hand, enhanced cell differentiation by BA in leaves promoted secondary metabolite accumulation. Thus, both root weight and leaf CPT concentration were positvely correlated.

In this study, NAA supplementations from 0.5 to 4 mg l^{-1} reduced the height, leaf number, leaf length, SLW, total weight, stem weight, and leaf weight of *C. acuminata* seedlings relative to the NAA control. It is possible that NAA applications increased auxin levels in seedlings beyond their optimal levels seen in the controls. NAA application at 0.5 mg l^{-1} increased root weight. Explanation for this result might be that NAA applications were affecting seedlings and a low NAA level produced normal and long roots whereas high NAA levels produced abnormal and short roots as observed in our experiment.

In many other studies, auxins have been shown to inhibit alkaloid biosynthesis and accumulation. For example, in *C. roseus* cell cultures, auxins exerted a dramatic inhibition on alkaloid terpenoid precursor availability. During one subculture, auxin depletion greatly enhanced ajmalicine content (Gantet and others 1997). Auxins have also been

shown to down-regulate gene expression in alkaloid biosynthesis. In *C. roseus* cell cultures the *sss* gene (for strictosidine synthase) and *tdc* gene (for tryptophan decarboxylase) were rapidly down-regulated by auxin (Pasquali and others 1992). Omission of NAA from *C. roseus* cell culture resulted in the accumulation of *tdc* mRNA. The addition of NAA, IAA, or 2,4-D rapidly reduced *tdc* transcript levels. Thus, one of the mechanisms that control the activity of terpenoid indole alkaloid biosynthesis in *C. roseus* was the negative regulation by auxin of the genes involved in the biosynthesis (Goddijn and others 1992). However, our studies showed that NAA applications did not affect leaf CPT concentration. It is possible that auxin had no effect on CPT biosynthesis.

The final total leaf CPT yields decreased with increasing NAA concentrations with the highest total leaf CPT yield in the NAA control. Leaf CPT concentrations were similar across the NAA treatments, but total leaf biomass decreased with increasing NAA concentrations. Our results suggest NAA applications decrease total leaf CPT yield. In the NAA study, significantly negative correlations were found between leaf number and leaf CPT concentration, leaf length and leaf CPT concentration. The study of the relationship between leaf length and leaf CPT concentration did not examine the same leaves, but the relationship was examined on the same seedling and was used to represent the same seedling. Our findings may suggest that a trade-off between plant growth (such as leaf number increment and leaf elongation) and secondary metabolite accumulation exists. For example, improved alkaloid accumulation was found to be related to a slower overall growth rate in suspended *Papaver somniferum* culture (Siah and Doran 1991). CPT concentration usually increases as a consequence of environmental stress (Liu and others 1997; Liu 2000), whereas plant growth decreases as a consequence of environmental stress. So both are negatively correlated.

In conclusion, increasing BA concentrations from 0 to 3 mg l^{-1} in growth media decreased the height, stem weight, and total leaf weight, but increased root weight of *C. acuminata* seedlings in a hydroponic culture system. High BA levels (1 and 3 mg l^{-1}) increased leaf CPT concentrations, but had no effect on total leaf CPT yield. There was a positive correlation between root weight and CPT concentration in leaves under BA treatments. Exogenous NAA supplementations from 0.5 to 4 mg l^{-1} reduced the height, leaf number, leaf length, SLW, total weight, stem weight, and leaf weight of *C. acuminata* compared with the NAA control. NAA applications had no effect on leaf CPT concentration and NAA applications decreased total leaf CPT yield. In addition, the effects of NAA applications from 0.5 to 4 mg l^{-1} on plant height, leaf length, and SLW were concentration independent. There were negative correlations between leaf number and leaf CPT concentration, between leaf length and leaf CPT concentration under NAA treatments.

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