Variation in camptothecin content in *Camptotheca acuminata* leaves

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Abstract. Variations in camptothecin (CPT) content in relation to leaf characteristics namely leaf area, leaf biomass, and specific leaf area (leaf area divided by leaf biomass), and the influence of climatic factors on CPT content in saplings of *Camptotheca acuminata* Decne. were studied. Leaf area and CPT content showed a logarithmic relationship in young leaves similar to that between leaf biomass and CPT content, while CPT content was linearly correlated to specific leaf area. There was no apparent relationship between each leaf characteristic and CPT content in old leaves. CPT contents in sapling leaves showed large variation from May to September and were highly correlated to the variation in the level of climatic factors, where high average temperature, high evaporation capacity and low precipitation increased CPT content according to stepwise regression analysis. This suggests that adverse growing conditions can induce CPT accumulation to enhance chemical defense in *C. acuminata*. Young leaves with area of 70 cm^2-100 cm^2 gave the highest quantity of CPT per leaf, thus the optimal harvest period would be when young leaves expanded to these sizes. In addition, sample collection on dry days is preferable as it increases CPT content in raw materials.

Keywords: Camptothecin; *Camptotheca acuminata* leaves; CPT optimization.

Introduction

Camptothecin (CPT) is a monoterpenoid indole alkaloid originally isolated from *Camptotheca acuminata* Decne, a deciduous tree native to south China, that has gained great attention for its significant antitumor activities in experimental studies (Wall et al., 1966). Irinotecan (CPT-11) (Masuda et al., 1992; Abigeres et al., 1995; Bleiberg, 1999) and topotecan (TPT) (Lilenbaum et al., 1995; Romanelli et al., 1998; Clements et al., 1999), two water-soluble derivatives of CPT, have gained approval by the Food and Drug Administration of the United States of America (FDA) for treating colorectal and ovarian cancer. Other camptothecins–such as 9-aminocamptothecin (9AC), 9-nitrocamptothecin (9NC), and 7-(4-methyl piperazino-methylene)-10,11-ethylenedioxycamptothecin (GG211) –have also showed remarkable potential in the treatment of carcinoma (Wall and Wani, 1996; Giovanella, 1997; Jeha et al., 1998; Stevenson et al., 1999). Camptothecins are lauded as one of the most promising anticancer drugs of the twenty-first century (Li and Adair, 1994).

CPT acts as a defensive chemical in *C. acuminata* though there is not much research on this aspect of CPT. It was reported that goats feeding on *C. acuminata* leaves became poisoned (Cao et al., 1986) as well as honeybees (Xia, 2000). In addition, CPT is strongly toxic to other animals. CPT is water-insoluble, but it and related analogs contain an α-hydroxy-δ-lactone ring functionality which hydrolyzes under certain conditions, i.e. at pH 7 or above, with the lactone moiety readily opening up to yield the water-soluble carboxylate form (Burke, 1996) which is highly toxic to animals and humans. Extensive toxicological and pharmacological studies in mice, dogs, and pigs determined that CPT is toxic due to inflammatory ileitis and myelo suppression (Giovanella, 1997). Therefore, CPT plays a role in deterring herbivore feeding in *C. acuminata*. Thus understanding the variation in CPT content in *C. acuminata* will increase our knowledge of the regulation of chemical defenses, as well as help to optimize harvest opportunities for the raw materials of this important anti-cancer pre-drug.

CPT is present in all organs of *C. acuminata* (Lin et al., 1977). Traditionally, CPT has been extracted from root, root bark, and fruits (Lin et al., 1977), but recent results have indicated that CPT exists at high levels in very young leaves, even higher than in the fruits (Lopez-Meyer et al., 1994; Zhang and Yang, 1997). In this paper, the production of CPT is related to leaf characteristics–namely leaf area, leaf biomass, and specific leaf area (leaf area divided by leaf biomass)–and the influence of climatic factors in CPT content are all investigated. Additionally, based on the findings, an optimal scheme for harvesting of *C. acuminata* leaves for CPT extraction is discussed.

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Table 1. Geographical and meteorologic parameters for seed sources of *C. acuminata*.

Seed source	Location	Geographical and meteorologic parameters					
		Latitude	Longitude	Elevation (m)	Annual mean temperature (°C)	Annual evaporation capacity (mm)	Annual precipitation (mm)
SC	Jintang, Sichuan Province	30.72°N	104.61°E	507.1	16.7	1169.1	926.4
YN	Jingdong, Yunnan Province	24.28°N	101.05°E	1162.3	18.3	1743.5	1087.0
SX	Wugong, Shaanxi Province	34.15°N	108.13°E	447.8	12.9	1505.3	631.0
GD	Lianxian, Guangdong Province	24.47°N	112.23°E	97.6	19.5	1419.1	1571.8

Materials and Methods

Cultivation Conditions

Saplings were grown from seeds collected in January 1997 from four different geographical sources of *C. acuminata* (Table 1). Seeds were sown at the Harbin Experimental Forest Farm of Northeast Forestry University, Harbin, China (126°37' E, 45°41' N) in March 1997. The site has an annual precipitation of 523.3 mm, an annual evaporation capacity of 1508.7 mm, and an annual mean temperature of 3.6°C. The coldest month is January, and the warmest month is July. Seedlings were transplanted to pots (diameter 10 cm, depth 8 cm) in May 1997, and subsequently transplanted to larger pots every several months as the plants grew bigger. From April to October, the seedlings were grown outdoors; otherwise they were kept in a greenhouse. No supplemental fertilizers were used. In the first growing season, they were coppiced twice (in July and September), and then four times (in March, May, July and October) every other growing season to enhance branching. Saplings were transplanted to the ground in April 2000. For the first 15 days, they were irrigated twice a day and once during the following 10 days. Spacing of trees ranged from 1.0-1.5 m between and within rows.

Sample Collections

To assess variations in CPT content in relation to leaf characteristics, young leaves and old leaves with different areas were randomly selected from each SC sapling in July 1999. (Young leaf was defined as any leaf located between the apex and the largest leaf on the same branch. Otherwise it was old leaf.) Each leaf was assayed as one sample.

To analyze the influence of climatic factors on CPT content, six young leaves from different saplings with area of 80-90 cm² were randomly selected from all four sources of saplings about every 15 days from May 29 to September 5, 2000. Each leaf was analyzed as one sample. Climatic data were obtained from Harbin Meteorological Administration, P. R. China.

Extraction and Analysis

Leaf area of each leaf were measured by a LI-3000A Portable Area Meter (LI-COR Co., USA), and then dried at 70°C to constant weight. Dried samples were ground in a mortar and stored in a desiccator.

About 0.1 g of dried leaf powder was put into a 5 mL volumetric flask, to which 4 mL of 61% ethanol was added. The mixture was extracted at 50°C for 10 min with ultrasonication (Branson Cleaning Equipment Co., USA.) (Yan et al., 2002). After cooling down to room temperature, 61% ethanol was added to 5 mL. One mL of the extract was centrifuged at 12,500 *g* for 10 min at 20°C (Model 22R Biofuge, Heraeus Instruments, Germany) and the supernatant analyzed for CPT content.

Determination of CPT content was performed with a high-performance liquid chromatography system (JASCO Inc., Japan) consisting of two Model 1580 pumps a Model 1575 UV detector, and a Techsphere ODS column (25 cm × 4.6 mm, 5 µm, HPLC Technology, U.K.). The HPLC conditions were: 254 nm as the detected wavelength, 1 mL/min as the flow rate, and 10 µL sample loop. The solvent gradient program was as follows: 25% acetonitrile/water increasing linearly to 50% acetonitrile/water in the first 15.0 min; then 50% acetonitrile/water isocratic for 3.0 min; beginning at 18 min, 50% acetonitrile/water decreasing linearly to 25% acetonitrile/water in 1.0 min; at 20.0 min, the program stops (ready for next injection). A CPT standard sample was supplied by The Stehlin Foundation for Cancer Research (Houston, TX, USA). The retention time of CPT was 10.7 min. CPT content was expressed as percent of dry weight.

Results and Discussion

CPT Distribution in Leaves

There was a logarithmic relationship between the leaf area and CPT content in young leaves of the SC saplings. When a leaf was smaller than 50 cm², CPT content plunged as the leaf enlarged, then the decrease slowed. As an example, CPT content was 0.41% with the leaf area at 2.61

cm^2; it decreased to 0.24% at 8.67 cm^2, 0.12% at 37.84 cm^2, and 0.04% at 116.02 cm^2 (Figure 1A). Besides leaf area, a similar connection appeared between leaf biomass and CPT content in young leaves (Figure 1B). CPT content diminished linearly as the increment of specific leaf area increased (Figure 2), implying that specific leaf area increases with leaf expansion (Figure 3).

In contrast to young leaves, no evident correlation was displayed between CPT content and leaf area (Figure 4), leaf biomass, or specific leaf area in old leaves of *C. acuminata*. CPT content fluctuated between 0.02%–0.06% in old leaves. Interestingly, the average CPT content of 0.04% corresponds to the lowest content in young leaves (0.03%), suggesting that CPT metabolism equilibrates or stops when the leaf turns old.

In general, vulnerable tissues and organs are defended more than senescent ones; many seeds, seedlings, buds and young tissues either sequester or synthesize large amounts of defense chemicals. The production of CPT in *C. acuminata* showed a similar pattern in our study. The highest CPT content was present in the youngest leaves, up to tenfold more than in old leaves, which may represent a juvenile chemical defense strategy in *C. acuminata*, as suggested by the findings on some other species (Turner, 1995).

Influence of Climatic Factors on CPT Accumulation

Variations in CPT content in the SC, YN, SX and GD *C. acuminata* saplings were similar from May to September 2000 (Figure 5). There were two peak contents, and except for the YN saplings, the first max CPT content was higher than the second, but no obvious second peak in the SX saplings appeared. The minimum CPT contents were all shown in the samples collected on July 9 for all saplings.

Comparing CPT contents with climatic data from Harbin during this period revealed some consonance between them. The lowest CPT content on July 9 corresponded to a period of maximum precipitation. In contrast, the two maximum contents were found during the lower levels of precipitation (cumulative precipitation data for 5 to 25 days before the date sample collection, Figure 6). In addition, the regression equation obtained by stepwise regression
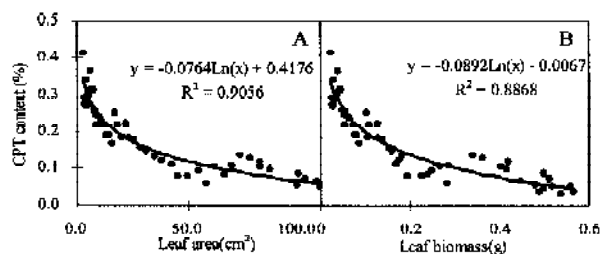
Figure 2. Correlation between specific leaf area and CPT content in young leaves of 4 year-old SC *C. acuminata* saplings. Each point represents the value for one leaf.

Figure 3. Relationship between specific leaf area and leaf area in young leaves of 4-year-old SC *C. acuminata* saplings. Each point represents the value for one leaf. Specific leaf area was leaf area divided by leaf mass.

Figure 1. Relationship between leaf area or leaf biomass, and CPT content in 4-year-old SC *C. acuminata* saplings. Each point represents the value for one leaf.
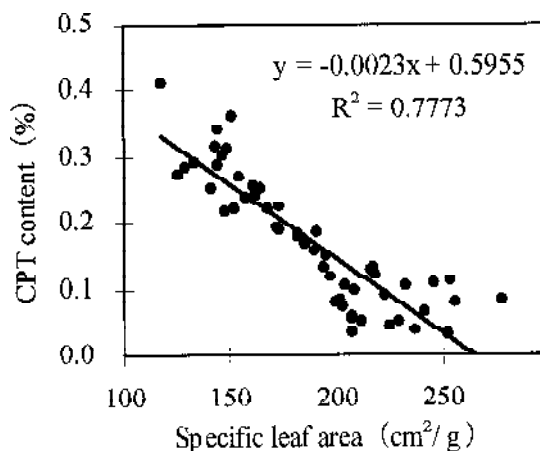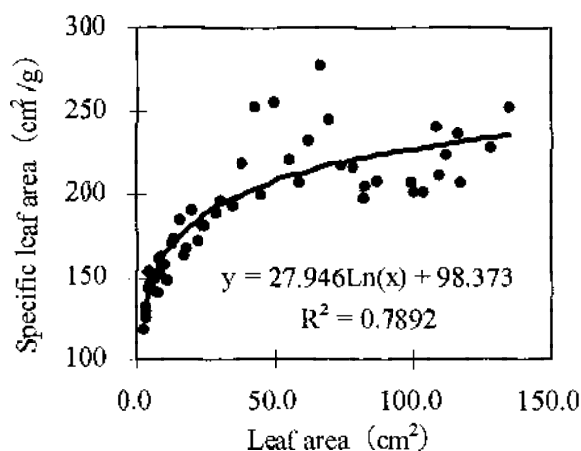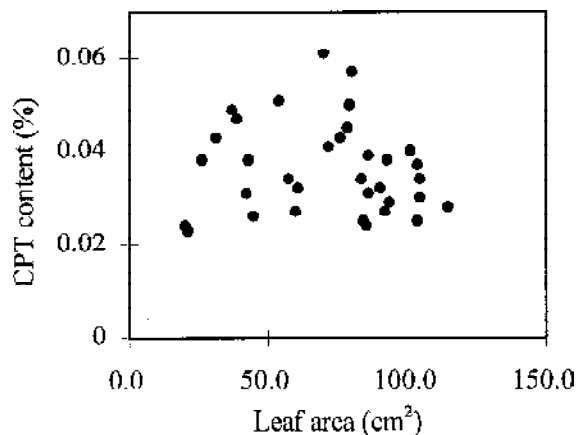
Figure 4. Relationship between leaf area and CPT content in old leaves of 4-year-old SC *C. acuminata* saplings. Each point represents the value for one leaf.

analysis–using the average air temperature, evaporation capacity, and precipitation data as three independent variables and CPT content as a dependent variable (Table 2)—showed a high correlation among climatic factors and CPT content. According to the analytical results, the influence of each factor on the CPT content of four different seed sources was similar (Figure 7). Basically, higher average air temperature and lower precipitation seems to promote

a higher CPT content. As for evaporation capacity, it seems conducive to CPT accumulation.

Usually, chemical defense can be induced by biotic and abiotic factors. Plants produce more chemical defense agents to protect themselves under stress since growth is slower and biomass loss by damage becomes worse under these conditions than under regular conditions. *Camptotheca acuminata* may also have an induction
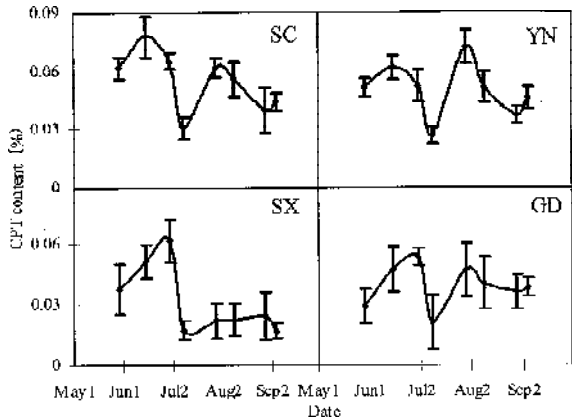

Figure 5. Variations in CPT content in 4-year-old *C. acuminata* saplings from four different seed sources from May to September 2000. Vertical bars originating from each data point represent the standard error of the mean (n=6).
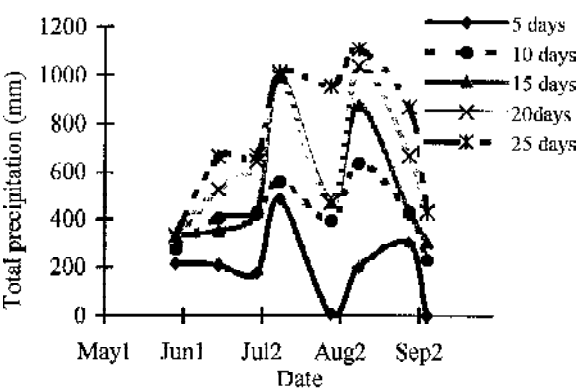

Figure 6. Total precipitation of each collection day in Harbin, PRC. The accumulation of precipitation data is shown for the 5-25 days before the date of collection.

Table 2. Stepwise regression analytical results among climatic factors and CPT content in 4-year-old *C. acuminata* saplings from four different sources from May to September 2000 in Harbin, PRC.

Source	Days	Regression equation	R^2
SC	5	$Y=0.0614537+0.0003568X_1^2-0.0000676X_1X_2+0.0000588X_1X_3+0.0000029X_2^2-0.0000037X_2X_3-0.0000004X_3^2$	0.9998
	10	$Y=-0.5800835+0.0007073X_2+0.0015289X_3-0.0004066X_1^2+0.0000286X_1X_2-0.0000002X_2^2-0.0000022X_2X_3$	0.9990
	15	$Y=-0.3427918+0.0003696X_2+0.0006486X_3+0.0000053X_1X_2+0.0000023X_1X_3-0.0000001X_2^2-0.0000003X_2X_3$	0.9974
	20	$Y=4.9011030-0.0067559X_2-0.0003263X_3+0.0000363X_1X_3+0.0000023X_2^2-0.0000002X_2X_3-0.0000002X_3^2$	0.9968
	25	$Y=0.8245602-0.0009128X_2+0.0000064X_1X_3+0.0000003X_2^2-0.0000001X_2X_3$	0.5507
YN	5	$Y=0.1657806-0.0131805X_1+0.0003673X_1^2-0.0000002X_3^2$	0.7721
	10	$Y=-0.4502043+0.0005026X_2+0.0012764X_3+0.0004458X_1X_2-0.0000352X_1X_3-0.0000011X_2X_3+0.0000003X_3^2$	0.9998
	15	$Y=-0.5241449+0.0244334X_1+0.0004590X_2-0.0000197X_1X_2+0.0000063X_1X_3-0.0000001X_2X_3-0.0000001X_3^2$	1.0000
	20	$Y=-10.0688090+0.0994900X_1-0.0014070X_1^2-0.0000211X_1X_2+0.0000002X_2^2-0.0000002X_2X_3+0.0000002X_3^2$	0.9998
	25	$Y=-2.0003853+0.0931481X_1+0.0010068X_2+0.0003980X_1^2-0.0000508X_1X_2+0.0000001X_2X_3-0.0000002X_3^2$	0.9926
SX	5	$Y=0.0579609-0.0000050X_1X_2+0.0000115X_1X_3-0.0000006X_3^2$	0.7237
	10	$Y=-0.8481723+0.0550806X_1+0.0005376X_2+0.0005285X_3-0.0009482X_1^2-0.0000232X_1X_2-0.0000006X_3^2$	0.8334
	15	$Y=-0.9077752+0.0376611X_1+0.0007730X_2+0.0004051X_3-0.0010185X_1^2-0.0000003X_2^2-0.0000003X_3^2$	0.9783
	20	$Y=3.3331198-0.0210205X_1-0.0042460X_2-0.0005109X_3+0.0000631X_1X_3+0.0000014X_2^2-0.0000006X_3^2$	0.9942
	25	$Y=-0.4003720+0.096156X_1-0.0007724X_2-0.0003785X_1^2-0.0000372X_1X_2+0.0000004X_2^2-0.0000001X_2X_3$	0.9779
GD	5	$Y=-0.1773299+0.0104491X_1+0.0002347X_2+0.0004921X_3-0.0000130X_1X_2-0.0000183X_1X_3-0.0000002X_3^2$	0.9767
	10	$Y=-0.5961121+0.0178596X_1+0.0006435X_2+0.0010856X_3-0.0000505X_1X_3-0.0000004X_2^2+0.0000001X_3^2$	0.9797
	15	$Y=0.2048370+0.0309537X_1-0.0024383X_3-0.0023671X_1^2+0.0001650X_1X_3-0.0000012X_3^2$	0.9220
	20	$Y=2.9489770-0.0039205X_2-0.0006285X_3-0.0003091X_1^2+0.0000515X_1X_3+0.0000013X_2^2-0.0000004X_3^2$	0.9685
	25	$Y=-20.0501959+1.063071X_1+0.0008940X_2+0.0000769X_3-0.0003758X_1^2-0.0000419X_1X_2-0.0000001X_3^2$	0.9884

Y: CPT content/ %; X_1: average temperature/ °C; X_2: evaporation capacity/ mm; X_3: precipitation/ mm; Days: data accumulated days, temperature data were averaging temperatures for some days before collection day; evaporation capacity and precipitation data compiled by summing evaporation capacity and precipitation of each day for some days before collection day.

mechanism to produce CPT. Liu et al. (1997) have shown that shading can induce a higher CPT content. Our findings also imply an induction mechanism. The optimal conditions for the growth and development of *C. acuminata* are warm and humid, and it often grows by brooklets in south China. It was much drier in Harbin than in their natural habitat during this study. Water-stress led to higher CPT content in *C. acuminata* leaves; otherwise abundant rainfall favors the growth of *C. acuminata* rather than CPT accumulation. Similarly, a higher average temperature and evaporation resulted in low humidity during this period in Harbin, promoting CPT accumulation instead of plant growth. Other related research also suggests an inducible mechanism for CPT production in *C. acuminata*. Two tryptophan decarboxylase (TDC) genes, encoding a key enzyme in CPT biosynthesis, *tdc1* and *tdc2*, were characterized from *C. acuminata*. The former was developmentally regulated while the latter was only expressed after induction conditions, suggesting a regulated and an induced mechanism for the production of CPT in *C. acuminata* (Lopez-Meyer and Nessler, 1997).

Harvest Optimization

CPT is an important, naturally occurring compound for the semi-synthetic CPT drugs, so the optimal harvesting

scheme for achieving maximum CPT yield is crucial in the cultivation of *C. acuminata*. Though very small, young leaves have a high CPT content, harvesting them is not desirable because of the low biomass yield. Moreover, over-harvesting of such young leaves may be harmful to the ontogenesis of plants, which affects the sustainable production of plant materials. A small, young leaf with high CPT content had a lower CPT yield. However, a young leaf with an area of 70 cm² -100 cm² (leaf biomass about 0.3 g-0.5 g) had the highest CPT yield per leaf (Figure 8). Optimal harvesting would, therefore, involve picking young leaves as they expanded to these sizes instead of collecting them all at the same time. The CPT yield in old leaves increased linearly with the leaf area and leaf biomass (Figure 9), which meant CPT yield in old leaves was not dependant on the time of collection but correlated to the quantity of leaves.

In addition, it is preferable to collect leaves during dry days to increase CPT yield.

Conclusion

CPT content in young leaves was much higher than in old leaves and was logarithmically related to leaf area and leaf biomass while suggesting a juvenile chemical defense strategy. Variations in CPT content were also induced by climatic factors. Higher average air temperature and evaporation capacity, or lower precipitation favored CPT production from May to September in Harbin, China and conducted that there was an induction mechanism to produce CPT in *C. acuminata*.

In addition, optimal harvest conditions would involve picking young leaves when they expanded to a leaf area of 70 cm² -100 cm² on sunny days instead of collecting them all at the same time.
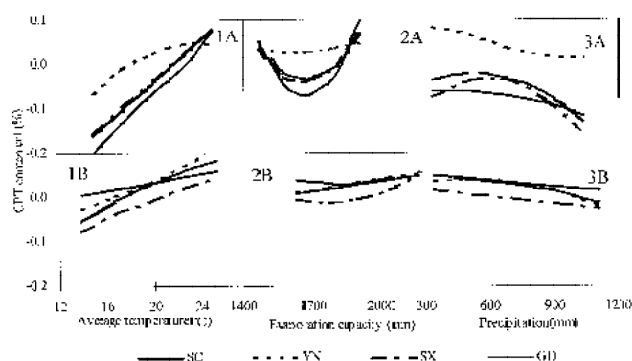
Figure 7. Influence of climatic factors on CPT content in 4-year-old *C. acuminata* saplings from four different seed sources according to stepwise regression analytical results. A: curve shows results of 20 days accumulated climatic data; B: curve shows results of 25 days accumulated climatic data.
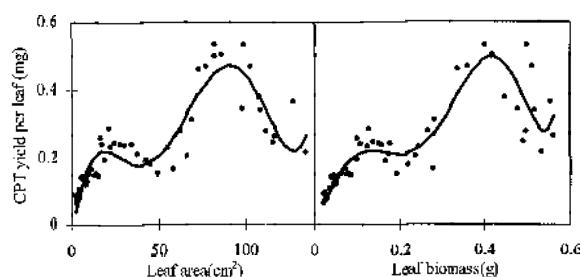

Figure 8. Relationship between CPT yield and leaf area and leaf biomass in young leaves of 4-year-old SC *C. acuminata* saplings. Each point represents the value for one leaf.
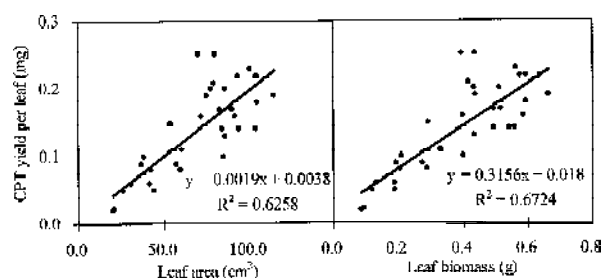

Figure 9. Correspondence of CPT yield to leaf area and leaf biomass in old leaves of 4-year-old SC *C. acuminata* saplings. Each point represents the value for one leaf.

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70 cm²~
100 cm²   0.3 g~0.5 g