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Traffic Pollution Influences Leaf Biochemistries of *Broussonetia papyrifera*

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Paper mulberry (*Broussonetia papyrifera*) is one of multifunctional species in agroforestry systems as well as one of traditional forages in many countries of Asia. Fully expanded tender leaves of *B. papyrifera* wildly growing under two traffic densities (a high traffic loads bearing more than 1000 vehicles per hour, HT; and a relatively clear section with almost no traffic loads, NT) were collected for carbohydrates, amino acids and phytohormones analysis. Leaves exposed to traffic pollutants were revealed to have significant lower amounts of carbohydrates and total amino acids than those growing at relatively clear environment. The levels of abscisic acid in the leaves significantly increased, while gibberellin acid, indoleaetic acid, and zeatin riboside in the leaves significantly decreased, with the traffic densities. The results indicated that the contents of carbohydrates, amino acids and phytohormones in the leaves of *B. papyrifera* could be adversely affected by traffic pollution. Variations of the leaf biochemistries of *B. papyrifera* exposed to traffic pollutants implied that *B. papyrifera* could physiologically regulate itself to adapt or resist traffic stress.

Keywords: Amino Acids; *Broussonetia papyrifera*; Carbohydrates; Phytohormounes; Traffic Pollutants

Introduction

Rapid development of livestock is bringing huge demands for forages globally. During the last few years, public concerns regarding food safety have intensively increased as a consequence of the increasing prevalence of some fatal diseases (e.g. *Salmonella enteritidis* in meat products and *Escherichia coli* 0157: H7 in beef) endangering human health. Frequent misuse of antibiotics, antibacterial, vitamins, hormones and the additive of some trace metals in animal feedstuffs also brought potential risks on human being. Controlling of hazard materials into livestock forages is one of internationally important issues for public health. Development of high quality plant forages has been prompted to avoid infectious agents into the animal feedstuffs and thus to strengthen the bio-security of humans (Martínez et al., 2005).

Paper mulberry (*Broussonetia papyrifera*) is a fast growing tree or shrub of the Moraceae family. This species commonly-naturally grows in various environments in Asia and Pacific countries (Malik & Husain, 2007) with large biomass and rapid propagation by shoot regeneration either from root or stem cuttings or seeding. It usually takes only 12 - 18 months to reach the harvest size of 3 - 4 m height (http://www.agroforestry.net/tti/Broussonetia-papermulb.pdf). Particularly, once have been harvested, the species could present faster growth rate and larger biomass than newly planted ones. Since the ancient time, *B. papyrifera* was widely used as multifunctional species in agroforestry ecosystems, e.g. manufacturing high-quality papers, cloths, and ropes (Liao et al., 2006), treating diseases as one of traditional Chinese medicines (Lee et al., 2001).

Differing from a variety of woody species used for furniture and manufacturing, Paper mulberry has been traditionally used as forage of livestock in many mountainous regions in China for long history when their tender leaves and twigs were harvested from natural stands. With the globally rapid development of domestic livestock and the huge demands for forages, values of wild plant resources such as mulberry have been intensively concerned (Hibib, 2004). Recently, farmers in mountainous provinces of China (e.g. Hubei, Guangxi and Jiangxi) have been encouraged to grow large area of Paper mulberry as a cash crop. Based on the literature survey, research on this species mainly focused on medicinal properties (Kwak et al., 2003), bark yield (Saito et al., 2009), tissue culture and rapid propagation (Li et al., 2008), influence on native scrub forest (Malik & Husain, 2007) and efficiency of heavy metals removal (Nagpal et al., 2011). Being one of traditional fodder shrubs with high levels of crude protein, minerals and digestibility in the leaves and twigs, *B. papyrifera* has been less investigated on the changes in biochemistries under the exposure of traffic exhaust. Researchers have demonstrated that pollution did depress the properties of fodder trees (shrubs) (Sanz et al., 2011). Automobile exhaust gas, a dominant cause of atmospheric pollution in urban and rural areas owing to the increasing number of vehicles, could be transported from urban to remote mountain areas (Sakugawa & Cape, 2007). In the present study, we detected the variations of carbohydrates, amino acids as well as phytohormones in the leaves of *B. papyrifera* exposed to traffic exhaust. The objectives were to examine the potential impacts of traffic pollution on the fodder properties, and to elucidate the mechanism by which this species responded to traffic stress.

Materials and Methods

Plant Sampling and Processing

Naturally-growing Paper mulberry shrubs were sampled from two environments with different traffic densities in Guangzhou city, southern China. The first one was selected along two freeways running along South China Botanical Garden with mean traffic loads of more than 1000 vehicles per hour. This section stood for the environment receiving high levels of automobile exhaust from point sources (high traffic loads, HT). The reference environment was selected in the botanical garden where vehicles were forbidden to enter. This section stood for the relatively clear environment without direct point source of traffic exhaust (NT). The two sampling sections, partitioned by the bounding wall of the garden, had similar soil property and climate condition.

Fifteen Paper mulberry shrubs with similar appearance and without visible injury in leaves were randomly selected from the different environment in November, respectively. All Paper mulberry shrubs were selected at least 500 m away from each other. The shrubs were all annual with about 9-month old. For each selected shrub, a composite sample with between 15 and 20 fully expanded tender leaves was taken from the outer canopy, stored in an icebox and carried back to the laboratory immediately.

In the laboratory, all the leaf samples from each section were divided into two parts. One part was freshly weighted and frozen in liquid nitrogen and stored at –20˚C for plant hormones analysis. The other part was washed thoroughly with distilled water, and dried at 60˚C for at least 48 hrs and ground using a mortar and pestle for later analysis.

Air Quality Monitoring

Ambient air quality was monitored from early-November to mid-December for total suspended particulates (TSP), particulate matter less than 2.5 microns in diameter ($PM_{2.5}$), sulphur dioxide (SO_2) and nitrogen oxides (NO_x including NO and NO_2) with moderate-volume sampler (TH-150CIII, China) located at 1.5 m above ground level. Total suspended particulates and $PM_{2.5}$ were trapped on quartz fibre filters (tare weighted before sampling) attached to the hopper of the samplers operated continuously for 24 hrs. After sampling, the filters were stored in a desiccator with constant temperature for at least 24 hrs and re-weighted with a precision balance. The concentrations of SO_2 were determined by absorbing air (0.5 $L \cdot min^{-1}$ for 45 - 60 mins, 5 replicates) into a buffering solution of formaldehyde, which was later analyzed through a pararosaniline spectrophotometry (SEP-HJ482, 2009). Nitrogen oxides were absorbed (0.4 $L \cdot min^{-1}$ for 45 - 60 mins, 5 replicates) by N-ethylene diamine dihydrochloride and then were determined spectrophotometrically (SEP-HJ479, 2009). The data were presented as 24 hrs average concentrations, and expressed as $\mu g \cdot m^{-3}$.

Carbohydrate Analysis

Approximately 50 mg of the oven-dried leaf powder of each sample was extracted with 80% ethanol (v/v) at 85˚C for 1 h. The solutions were then centrifuged at 12000 g for 10 mins. The ethanol extraction step was repeated three times. The three resulting supernatants were combined, treated with activated charcoal, and evaporated to dryness in a vacuum evaporator. The residues were redissolved in distilled water, and subjected to soluble sugar analysis using the anthrone-sulfuric acid method (Ebell, 1969). Following removal of soluble sugars, the remaining pellets were oven-dried overnight at 60˚C and retained for starch analysis according to the procedures described in previous publications (Vu et al., 2002). Total nonstructural carbohydrates (TNC) were calculated as the sum of soluble sugar and starch. Cellulose content was determined by the method of Updegraff (1969). All the analyses were repeated five times.

Determination of Amino Acids

An amount (100.0 - 200.0 mg) of leaf powder was placed in a hydrolysis tube. Each sample had 3 parallel repetitions. The hydrolysis tube was added in 15 mL of 6 M hydrochloric acid (HCl) and 1.0 mL of 1% mercaptoacetic acid, then sealed and heated in vacuum at 110˚C for 22 hrs. After hydrolysis, the hydrolysis solution was filtered into a 50.0 mL volumetric flask. The dilute solution (1.0 mL) was transferred into a 25.0 mL beaker in vacuum, and vaporized. Repeat this process once again by adding 1.0 - 2.0 mL of distilled water. Finally, the residual was dissolved in 1.0 mL of 0.02 M HCl, and filtered through a membrane (0.22 μm). The solution was used to determine the contents of aspartic acid (Asp), threonine (Thr), serine(Ser), glutamic acid (Glu), glycine (Gly), alanine (Ala), cystine (Cys), valine (Val), methionine (Met), isoleucine (Ile), leucine (Leu), tyrosine (Tyr), phenylalanine (Phe), lysine (Lys), histidine (His), arginine (Arg) and proline (Pro) by Amino Acid Analyzer (L-8800, Hitachi, Japan). The analysis was carried out according to the standard analytical procedures proposed by Chen et al. (2008).

Determination of Leaf Hormones

The extraction, purification and determination of endogenous levels of indoleaetic acid (IAA), gibberellin acid (GA), abscisic acid (ABA) and zeatin riboside (ZR) by an indirect enzyme-linked immunosorbent assay (ELISA) technique were performed as described by Zhao et al. (2006). The samples were homogenized in liquid nitrogen and extracted in cold 80% (v/v) methanol with butylated hydroxytoluene (1 $mmol \cdot L^{-1}$) overnight at 4˚C. The extracts were collected after centrifugation at 10000 × g (4˚C) for 20 mins, the extracts were passed through a C18 Sep-Pakcatridge (Waters, Milford, MA) and dried in N_2. The residues were dissolved in PBS (0.01 $mol \cdot L^{-1}$, pH 7.4) in order to determine the levels of IAA, GA, ABA and ZR. Microtitration plates (Nunc) were coated with synthetic IAA, GA, ABA or ZR ovalbumin conjugates in $NaHCO_3$ buffer (50 $mmol \cdot L^{-1}$, pH 9.6) and left overnight at 37˚C. Ovalbumin solution (10 $mg \cdot mL^{-1}$) was added to each well in order to block nonspecific binding. After incubation for 30 min at 37˚C, standard IAA, GA, ABA, ZR, samples and antibodies were added and incubated for a further 45 min at 37˚C. The antibodies against IAA, GA, ABA and ZR were obtained as described by Yang et al. (2001). Then horseradish peroxidase-labelled goat antirabbit immunoglobulin was added to each well and incubated for 1 h at 37˚C. Finally, the buffered enzyme substrate (orthophenylenediamino) was added, and the enzyme reaction was carried out in the dark at 37˚C for 15 min, then terminated using 3 $mol \cdot L^{-1}$ H_2SO_4. The absorbance was recorded at 490 nm. Calculations of the enzyme-immunoassay data were per-

formed as described by Yang et al. (2001). In this study the percentage recovery of each hormone was calculated by adding known amounts of standard hormone to a split extract. Percentage recoveries were all above 90%, and all sample extract dilution curves paralleled the standard curves, indicating the absence of nonspecific inhibitors in the extracts. All the hormones were analyzed at College of Crop Science, China Agricultural University.

Statistical Analysis

The data were shown as mean ± standard deviation. Mean comparison was performed to test the differences between the two traffic loads at the confidence level of 95% by paired-samples T-test using software SPSS 10.0 (SPSS Inc., Chicago, IL, USA).

Results

Ambient Pollutants

Considering the main pollutants at the different environments, traffic exposure brought significantly higher concentrations of TSP, $PM_{2.5}$, NO_x, and SO_2 (**Table 1**). The ambient mean concentrations of gas pollutants at HT site were nearly 10 and 4 times higher than those at NT for NO_x and SO_2, respectively. Traffic emission deteriorated the ambient air quality at HT, thus gave the opportunity to compare the biochemistries in the leaf of *B. papyrifera* grown under the traffic exposure.

Carbohydrates Content

Exposure to traffic loads not only caused significant decrease of soluble sugars and total nonstructural carbohydrates (TNC), but also caused dramatic decrease of cellulose content in the leaves of *B. papyrifera* (**Table 2**). Total soluble sugars in leaves of *B. papyrifera* exposed to traffic pollutants decreased by approx 50% ($P < 0.01$) relative to those growing under relatively clear environment (NT). Considering leaf soluble sugars and starch together, the TNC content decreased by *c.* 46% ($P < 0.01$) in leaves at HT, despite starch contents were not significantly different between the sites. Noticeably, traffic exposure statistically decreased the content of some structural carbohydrates in *B. papyrifera* leaves ($P < 0.01$), e.g. cellulose decreased more than 26%, compared to those at NT. However, the decreased magnitude in cellulose contents was not as high as the ones in soluble sugars and TNC.

Table 1.
Comparison of the main pollutants between the environments with high traffic loads of more than 1000 vehicles per hour (HT) and with not traffics (NT). Data of total suspended particulates (TSP) and particulate matter less than 2.5 microns in diameter ($PM_{2.5}$) was 24-hour average value. Nitrogen oxides including NO and NO_2 (NO_x) and sulphur dioxide (SO_2) were shown as the mean and standard variation of 5 replication measurements. Data was presented as $\mu g \cdot m^{-3}$.

Pollutants	HT	NT
TSP	252.93 ± 80.40**	94.88 ± 12.00
$PM_{2.5}$	131.18 ± 41.35**	63.31 ± 18.75
NO_x	76.83 ± 20.74**	7.71 ± 2.59
SO_2	158.90 ± 23.34**	44.56 ± 9.09

**Extremely statistical difference between the environments with the values of $P < 0.01$.

Leaf Amino Acids

The individual and total amino acids in the leaves of *B. papyrifera* grown under different traffic densities were shown in **Table 3**. Among the detected 17 individual amino acids, Glu, Asp, Les and Lys were the most abundant amino acids while His, Cys and Met were the lowest ones accounting for about 40% and only 5% of total amino acids, respectively, in the leaves at both environments. Traffic exposure significantly

Table 2.
Carbohydrate contents ($mg \cdot g^{-1}$ of dry weight) in the leaves of *B. papyrifera* growing at the environments with high traffic loads of more than 1000 vehicles per hour (HT) and with not traffics (NT). Values given were mean ± standard deviation. Mean values (n = 15 samples) were compared by paired-samples T-test at the significant level of $P < 0.05$.

Contents	HT	NT
Soluble sugar	98.10 ± 24.12	205.07 ± 57.26**
Starch	12.64 ± 2.42	13.22 ± 1.44
Cellulose	169.05 ± 47.74	229.91 ± 28.99**
TNC	110.74 ± 24.11	217.52 ± 61.89**

**Extremely statistical difference between the environments with the values of $P < 0.01$.

Table 3.
Comparison of amino acids (% of dry weight) in the leaves of *B. papyrifera* growing at the environments with high traffic loads of more than 1000 vehicles per hour (HT) and with not traffics (NT). Values given were mean ± standard deviation. Mean values (n = 15 samples) were compared by paired-samples T-test at the significant level of $P < 0.05$.

Species	HT	NT
Glu	1.87 ± 0.10	2.09 ± 0.12**
Asp	1.74 ± 0.08	1.83 ± 0.09
Leu	1.33 ± 0.09	1.69 ± 0.07**
Lys	1.30 ± 0.08	1.50 ± 0.11**
Phe	1.17 ± 0.02	1.28 ± 0.11
Gly	0.98 ± 0.05	1.09 ± 0.06
Ala	0.96 ± 0.09	1.23 ± 0.10
Pro	0.90 ± 0.12	0.82 ± 0.16
Val	0.82 ± 0.01	0.98 ± 0.07**
Ile	0.81 ± 0.03	0.98 ± 0.04**
Tyr	0.80 ± 0.04	0.82 ± 0.03
Thr	0.79 ± 0.05	0.88 ± 0.06**
Ser	0.78 ± 0.02	0.87 ± 0.06
Arg	0.76 ± 0.14	1.06 ± 0.17**
His	0.47 ± 0.05	0.53 ± 0.03
Cys	0.22 ± 0.04	0.15 ± 0.02
Met	0.19 ± 0.04	0.23 ± 0.04
Total	15.89 ± 0.50	18.05 ± 0.89**

**Extremely statistical difference between the environments with the values of $P < 0.01$.

decreased the leaf total amino acids, with expectedly highest contents in the leaves from the relatively clear environment (NT). However, concentrations of Asp, Phe, Gly, Ala, Pro, Tyr, Ser, His, Cys, and Met did not respond to the presence of traffic.

Leaf Phytohormones

Facing to traffic exhausts, paper mulberry patterned distinguishingly for certain hormones in the leaves (**Table 4**). It could be easily observed that the plant hormones varied species-specifically in the leaves between the cases. There were significant increase in ABA and significant decrease in GA, IAA, and ZR in leaves exposed to traffic exhausts (HT) compared with those grown at the relatively clear environment (NT). Levels of IAA were revealed particularly affected by the traffic pollutants, with almost 3 times higher in the leaves at HT than at and NT.

Table 4.
The levels of phytohormones ($mg \cdot g^{-1}$ of fresh weight) in the leaves of *B. papyrifera* growing at the environments with high traffic loads of more than 1000 vehicles per hour (HT) and with not traffics (NT). Values given were mean ± standard deviation. Mean values (n = 15 samples) were compared by paired-samples T-test at the significant level of $P < 0.05$.

Contents	HT	NT
ABA	$68.86 \pm 1.26^{**}$	61.25 ± 1.09
GA	19.79 ± 0.42	$24.47 \pm 0.61^{**}$
IAA	37.33 ± 0.81	$117.03 \pm 2.18^{**}$
ZR	15.66 ± 0.26	$26.77 \pm 0.61^{**}$

**Extremely statistical difference between the environments with the values of $P < 0.01$.

Discussion

Partitioned only by the bounding wall of the botanical garden, the two sampling locations were considered with no significant difference in soil and climate properties. The influence of traffic pollutants was mainly discussed in this study. It's well known that vehicles could directly emit a large amount of TSP and $PM_{2.5}$, which could have significant effects on ambient quality (Kunzli et al., 2006). As revealed by Sakugawa et al. (2011), nitrite was a dominant source of photochemical formation of OH radical in both gasoline and diesel car exhausts. The atmospheric NO_2 concentration at the roadside in the forest was highly correlated with the traffic density of buses (Kume et al., 2009). In this study, the significantly high concentrations of TSP, $PM_{2.5}$, NO_x and SO_2 at HT indicated that automobile exhaust might have harmful effects on plant species (Shigihara et al., 2008). At the same time, high SO_2 in NT suggested that wind transported a considerable amount of pollutants from traffic site to the botanical garden.

As revealed by researchers that environmental stresses like heavy metal and air pollution could lead to major alterations in carbohydrate metabolism of plants by decreasing of maximum photosynthetic rate and stomata conductance in plant leaves, increasing ethylene emission, and reducing leaf longevity (Thomas et al., 2006; Devi et al., 2007; Kume et al., 2009; Sakugawa et al., 2011). Total content of carbohydrates (in particular soluble sugar and TNC) in leaves of forages added nutritive value to animals (Shewmaker et al., 2006). In this study, the significant decrease in soluble sugar, TNC and cellulose contents in the leaves of *B. papyrifera* affected by traffic exposure agreed with Tripathi and Gautam (2007) who found that even short duration of air pollution significantly decreased the soluble sugar contents. Traffic pollutants could adversely affect plant physiological and morphological characteristic directly or indirectly. Various physiological deteriorations of plant leaves were correlated with the NO_2 concentration (Kume et al., 2000). We speculated that the noticeable decrease of soluble sugar, NTC in the leave of *B. papyrifera* might be due to: 1) the inhibition of RUBP carboxylase activity caused by traffic exhausts, because RUBP carboxylase was a most abundant key enzyme in photosynthesis for carbohydrates assimilation in plants (Tripathi & Gautam, 2007). The decrease of RUBP carboxylase activity thereby resulted in reduced levels of carbohydrates; 2) the increased respiration and decreased CO_2 fixation because of chlorophyll deterioration caused by the traffic exhausts. Usually, plants could increase soluble sugar in leaves when assimilation rates were in excess of carbohydrate consumption rates; 3) the lower allocation of carbohydrates to cell walls or a decrease in the activity of cellulose synthase leading to the reduction of cellulose in the leaves of *B. papyrifera* exposed under traffic loads. We suggested that the leaf carbohydrate contents *of B. papyrifera* be indicators of traffic pollution for early nutritive diagnosis or as markers for physiological damage to forage prior to the onset of visible injury symptoms.

Amino acids were known to play a vital role in the osmotic adjustment and in the tolerance and detoxification of plants (Hall, 2002). For instance, Pro was revealed to be very important in ameliorating environmental stress in many higher plants (Wang et al., 2009). Another amino acid, His, also typically involved in metal stress tolerance (Sharma & Dietz, 2006). In this study, however, both of the two amino acids did not increase from NT to HT (**Table 3**). The similar levels of Pro and His found in the leaves of *B. papyrifera* collected from the two environments compared to other amino acids indicated that this species might be able to actively accumulate some amino acids, other than Pro and His, depending on the type of environmental stress. This finding was in agreement with Hussein and Terry (2002) who reported similar observations in some plants growing at crude oil contaminated saline environment, but in disagreement with other studies in which the concentration of Pro was found markedly increased under environmental stress despite of the decrease of total amino acids (Balestrasse et al., 2005). Cysteine, a SH containing amino acid, was a key constituent of phytochelatins and played an important role in metal detoxification. An increase in Cys content was recorded in leaves of plants irrigated with effluents (Chandra et al., 2009). However, Cys did not increase or decrease in the leaves of *B. papyrifera* between the traffic loads, implying that this species might detoxify by accumulating other amino acids. In the present study, *B. papyrifera* was found to biochemically-physiologically respond to the traffic pollution. Traffic exposure did decrease some individual amino acid (Glu, Leu, Lys, Val, Ile, Thr, Arg) as well as the total amino acids levels. This result was well consistent with numerous findings concerning plants facing to environmental stresses (Wang et al., 2009).

Plants could respond both physiologically and anatomically to environmental stresses usually under the regulations of plant hormones including ABA, GA, IAA, and ZR (Li et al., 2002).

Plant ABA was considered as an inhibitor of leaf growth and was suggested to be a regulator of leaf stomatal aperture (Jiang et al., 2003). In the present study, the significantly higher levels of ABA in leaves exposed to traffic exhaust implied that *B. papyrifera* might resist the traffic pollution by decreasing the leaf stomatal aperture and increasing leaf hydraulic conductivity. It was reported that high ABA levels found in leaves were generally consistent with low stomatal aperture and high hydraulic conductivity (Jiang et al., 2003). The patterns of leaf ABA in the present study were also revealed by Monni et al. (2001) who found plants growing near pollution source had higher contents of ABA in their stems compared to those growing farther.

Unlike the significant increase in IAA, GA, ZR and significant decrease in ABA in the leaves of *Arabidopsis thaliana* grown under the elevated CO_2 (Teng et al., 2006), plant hormones in this study were observed noticeably reduced in IAA, GA and ZR in the leaves grown at the traffic environment. GA and IAA could enhance plant growth and development by stimulating cell division, cell elongation and protein synthesis (Yong et al., 2000). The noticeable reduce of GA and IAA in the leaves affected by traffic exhausts implied that the growth of *B. papyrifera* might be adversely affected. We proposed that change in the levels of plant hormones probably was one of physiological responses regulating the adaptability or resistance of *B. papyrifera* growing under traffic pollution.

Conclusion

Traffic pollution significantly decreased the carbohydrates and total amino acids in the leaves of *B. papyrifera*, which might adversely decrease the nutritive values of this species. Concerns on this decrease should be arisen when the wild shrub was frequently and increasingly used as one of important plant forages. The variations of plant hormones in the leaves exposed to traffic pollutants implied that this species could physiologically and bio-protectively regulate itself to adapt or resist traffic pollution. The biochemistries could be used as indicators for early properties diagnosis.

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