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Biogenic volatile organic compound emissions from bamboo: Exploring patterns of diversity across species

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ABSTRACT

Emissions of biogenic volatile organic compounds (BVOCs) from plant leaves play significant roles in biological and atmospheric processes. BVOCs emissions can involve a diverse number of compounds and are an important method of plant signaling. However, some emissions of volatile compounds can negatively impact air quality at a regional scale. In order to better understand the role of BVOCs in plant physiology and chemical ecology, and to better predict how these emissions may alter air quality, the underlying relationships between these diverse compounds must be addressed. Comparisons between biogenic emissions from plants have been difficult in the past, as surveys have tended to focus on a limited number of compounds, and analytical techniques have lacked the ability to detect or separate compounds within functional groups. Additionally, closely related species typically emit similar compounds, making the reasons that plants emit certain BVOCs over others difficult to elucidate.

We have identified the bamboos as a novel system for studying BVOC emission because they emit a diverse range of compounds and emission of isoprene, a well-conserved compound, is widely variable across species. Between 75-196 individual compounds were identified using two-dimensional gas chromatography with time-of-flight mass spectrometry (GCxGC TOF-MS) from 12 species of bamboo, and one other species of grass. BVOCs emissions were analyzed by comparing patterns of compound class emission after assigning each compound to class based on its functional groups. Using non-metric multidimensional data scaling, we mapped the relationship between bamboo plants based on their compound class composition. We found significant differences in overall compound class composition between species that do and do not emit isoprene, suggesting there is a relationship between isoprene emission and the pattern of BVOC emissions observed in the bamboos. Overall, BVOC composition varies significantly amongst species of bamboo and may differentially impact chemical ecology and atmospheric chemistry.

Keywords
Biogenic volatile organic compounds, bamboo, isoprene, GCxGC TOF-MS, chemical ecology

Abbreviations
Biogenic volatile organic compounds (BVOCs)
Green leaf volatiles (GLVs),
International Union of Pure and Applied Chemists (IUPAC)
Non-metric multidimensional scaling (NMDS)
Photosynthetically active radiation (PAR)
Secondary organic aerosol (SOA)
Solid phase microextraction (SPME)
Two-dimensional gas chromatography with time-of-flight mass spectrometry (GCxGC TOF-MS)
Volatile organic compound (VOC)

INTRODUCTION

Carbon emissions from plants, known as biogenic volatile organic compounds (BVOCs), are a significant source of atmospheric carbon. BVOCs comprise 95% of the total global volatile organic compound (VOC) emissions (Loreto *et al.* 2008). Biogenic sources of carbon can be emitted in a wide range of structural forms with various degrees of volatility and

reactivity. Common plant-based biogenics include terpenes, alkenes, alkanes, alcohols, ethers, esters, and acids (Kesselmeier and Staudt 1999). Depending on their chemical structure, BVOCs may be long-lived or very reactive in the atmosphere.

Because of the magnitude and diversity of BVOCs generated in plant tissues, the release of these compounds can have significant impacts atmospheric chemistry. Ozone generation, secondary organic aerosol formation, and extended lifetimes of other pollutants can all occur as a result of BVOC emission (Arneth *et al.* 2008; Papiez *et al.* 2009). Tropospheric ozone levels can increase as a result of BVOC oxidation in the presence of nitrogen oxides. Ozone is known to have negative effects on human health, and causes damage to lung tissues in humans and animals and leaf tissues in plants (United States Environmental Protection Agency). Secondary organic aerosols (SOA), a form of particulate matter that influences regional visibility and temperatures, can be formed if larger BVOC molecules aggregate (Papiez *et al.* 2009). Atmospheric peroxides break down BVOCs, and depletions of peroxides in the presence of large amounts of BVOCs may extend the lifetime of other greenhouse gases in the atmosphere (Arneth *et al.* 2008).

Isoprene (2-methyl 1,3-butadiene, C_5H_8), a reactive molecule composed solely of carbon and hydrogen, is one of the most significant BVOCs. Isoprene is the most abundant non-methane BVOC, and 711 Tg y^{-1} are emitted from vegetation spanning a wide range of plant groups (Harley *et al.* 1999; Ashworth *et al.* 2010). The bond structure of isoprene makes it very reactive in the troposphere, where isoprene is quickly oxidized by peroxide radicals, leads to significant increases in tropospheric ozone, and ultimately broken down to CO_2 and water (Sharkey *et al.* 2008; Ashworth *et al.* 2010). Because isoprene is widespread, reactive, and can create by-products which are detrimental to human health, its emission has been well characterized in a number of model plant systems often associated with large-scale monocultures, including poplar, oak, and eucalyptus. Though emissions of isoprene are found in plant groups that are phylogenitically dispersed throughout the plant kingdom, it is typically well-constrained within a given plant group, making comparisons between the physiology of isoprene emitting and non-emitting plants difficult to obtain (Harley *et al.* 1999; Sharkey *et al.* 2008). Functionally, isoprene has been shown to increase with light and temperature, and has been hypothesized to help plants combat temperature and ozone stress, though the question as to why plants make isoprene still remains unanswered (Fortunati *et al.* 2008; Sharkey 2009).

The range of BVOCs emitted by plants extends beyond isoprene to structurally and functionally diverse categories of compounds that can play important roles in chemical ecology, plant-insect and plant-plant communication. When wounded, many plants emit green leaf volatiles (GLVs), some of which are responsible for the characteristic "fresh cut grass" smell of leaves. GLVs include a variety of oxygenated C_6 through C_8 compounds like aldehydes and alcohols. The presence of GLV emission is associated with physical damage to the lipid membranes of leaves as a result of stress or in response to herbivory (Holopainen 2004).

Compounds in the terpenoid family, of which isoprene makes up a single unit, are widely emitted by plants and are important signaling molecules. Monoterpenes ($C_{10}H_{16}$) and sesquiterpenes ($C_{15}H_{24}$), are fragrant compounds can exist in a number of structural forms which serve a variety of ecological functions (Kesselmeier and Staudt 1999; Duhl *et al.* 2008). Despite the importance and diversity of BVOCs, measurements of leaf-level emissions are typically constrained to a limited set of compounds due to availability of standards or sensitivity of instrumentation (Duhl 2008; Ortega and Helmig 2008). As a result, studies of emissions are constrained to compounds like isoprene and the potential combined effects of other BVOC compounds and their impacts on ecology or atmospheric chemistry are not considered.

We have identified the bamboos as a novel system for studying BVOC emission because, unlike most isoprene emitting plants, bamboos do not emit isoprene uniformly within their clade. A survey of isoprene emission in 75 species in 25 genera found that basal isoprene emission rates range from 0 – 47 nmol isoprene m^{-2} sec^{-1}

(Melnychenko and Rosenstiel, unpublished). This pattern of variation exists across genera and within a genus at the species and cultivar levels.

Because bamboos do not uniformly emit isoprene, we hypothesized that other emissions of BVOCs would vary within the clade as well. The full range of BVOC emissions in 12 bamboo species and one other grass species have been analyzed alongside isoprene emission and are presented here.

MATERIALS AND METHODS

Bamboo growth conditions

Twelve species of bamboo within six genera of the subtribe Bambusoideae were cultivated at Portland State University in the Research Greenhouse facility. Study species were chosen based on preliminary surveys of isoprene emission in bamboos and selected to represent a range of basal isoprene emission rates found in plants phylogenetically dispersed within the Bambusoideae. One member of Arundinoideae, *Arundo donax* var. 'Candy cane', was also included in this study. For some genera we selected multiple representative species within a genus that varied according to leaf characteristics or basal isoprene emission rate (Table 1).

A minimum of five plants per species were supplied by Bamboo Garden Nursery in North Plains, OR, and were transplanted into 10-15 gallon pots upon arrival to the facilities at Portland State University. Plants were grown at 22°C during the day, and 15°C at night for 8 months prior to this experiment. High-intensity discharge lamps were used from 6 am to 10 pm daily, and provided an average of 250 µmol photons m^{-2} sec^{-1} of photosynthetically active radiation (PAR) to the bamboo plants. Plants were watered every other day and fertilized with an organic nitrogen, phosphorus and potassium supplement once every three weeks.

Isoprene flux measurements

Measurements of *in situ* isoprene flux were made during August of 2011 from intact, attached leaves on greenhouse plants that were brought into the laboratory. A leaf was placed in the light-controlled cuvette (LI-6400, LiCor Inc. Lincoln, NE, USA) and was equilibrated at a flow of 200 µmol m^{-2} sec^{-1} at 1000 µmol

Table 1. Grass species used in this study for comparison of BVOC emissions. 'Habit' refers to the vegetative method of rhizome growth.

Genus and species rate	Subfamily	Abbreviation	Relative isoprene emission level	Basal isoprene emission rate (nmol isoprene m^{-2} sec^{-1})	Leaf color	Habit
Arundo donax	Arundinoideae	AdV	High	8.524	Variegated	Clumping
Arundinaria gigantea	Bambusoideae	AG	None	0.542	Green	Running
Bambusa ventricosa	Bambusoideae	Bve	High	6.244	Green	Clumping
Bambusa ventricosa 'Kimmei'	Bambusoideae	BveV	High	5.724	Variegated	Clumping
Fargesia rufa	Bambusoideae	Fr	None	0.410	Green	Clumping
Phyllostachys aurea	Bambusoideae	Pa	High	7.477	Green	Running
Phyllostachys edulis	Bambusoideae	Pe	None	0.864	Green	Running
Phyllostachys nigra	Bambusoideae	Pn	High	10.443	Green	Running
Pleioblastus chino	Bambusoideae	PLc	None	0.294	Green	Running
Pleioblastus chino 'Murakamianus'	Bambusoideae	PLcmV	None	0.876	Variegated	Running
Pleioblastus chino 'Vaginatus Variegatus'	Bambusoideae	PLcV	None	0.386	Variegated	Running
Sasa kurilensis	Bambusoideae	Sk	None	0.418	Green	Running
Sasa kurilensis 'Shimofuri'	Bambusoideae	SkV	High	2.136	Variegated	Running

photons m^{-2} sec^{-1} PAR for 10 minutes prior to sampling. Two milliliters of the effluent air stream was sampled from the cuvette using a syringe and then injected into a RGD2 Gas Chromatograph with Reducing Gas Detector. The isoprene peak was identified and quantified using an authentic standard.

BVOC sample collection

Eighty-four leaf samples were collected for BVOC emission profiling during the months of November and December 2010 from four individuals per species. Leaves that were third from the apex of a branch, in good condition and fully exposed to light were selected for this study. Individual leaves were cut at the petiole with an ultra-sharp razor one to three hours prior to sampling for BVOC analysis. Each leaf was placed in a clean 40 ml vial and capped with a new Teflon backed silicon septa. Samples in vials were purged for 4 minutes at a flow of 50ml min^{-1} with lab air passed through a hydrocarbon trap to scrub ambient VOCs.

"Dark" samples (62 leaves from 13 species): After sample vials were purged a clean, conditioned solid phase microextraction (SPME) fiber in an SPME assembly (Sigma-Aldrich, St. Louis, MO, USA) was inserted through the septa and the fiber was exposed to the leaf for 60 minutes. "Light" samples (21 leaves from 7 species): Individual samples were incubated under a cool light source set at 1000 µmol photons m^{-2} sec^{-1} PAR for 20 minutes. At the end of the light incubation period, the SPME fiber was inserted into the vial and exposed to the leaf for 40 minutes. After exposure to each leaf sample, the SPME fiber was inserted into the gas chromatograph injector for 10 minutes.

GCxGC TOF-MS

Two-dimensional gas chromatography with time-of-flight mass spectrometry (GCxGC TOF-MS) using a 4D Leco Pegasus GCxGC TOF-MS (Leco, St. Joseph, MI, USA). GCxGC TOF-MS allows compounds to be separated and represented in two dimensions; in the primary dimension compounds are separated within a column according to volatility or weight, in the secondary dimension compounds are separated according to their polarity. The TOF-MS detector allows for excellent qualitative identification of compounds based on their unique spectrum of fragment masses. A summary of GCxGC TOF-MS methodology and conditions used for BVOC analysis is given in Pankow *et al.* (2012). An example of a typical GCxGC TOF-MS chromatogram is shown in Figure 1.

Conditions were set up according to Pankow *et al.* (2012) with minor modifications. The injector was set at 225°C splitless injection, and for 3 minutes Helium carrier gas passed over the fiber and moved the sample into the column at a flow of 1 ml/min. The primary column was a DB-VRX, 45 m, 0.25 mm I.D., 1.4 m film (Agilent, Santa Clara, CA, USA). After samples travelled the GCxGC modulator employed a trap with cold gas from LN2, followed by a hot pulse at 20°C for release onto the secondary column, composed of Stabilwax, 1.5 m, 0.25 mm I.D., 0.25 mm film (Rested, Bellefonte, PA, USA). Each modulation occurred every 4 seconds, with a 0.9 second hot pulse between modulations. The GC oven was set at 45°C for 5 minutes, then stepped at 10°C /min to 175°C and was held at 175°C for 2 minutes, then stepped at 4°C /min to 240°C and was held at 240°C for 10 minutes. Each leaf

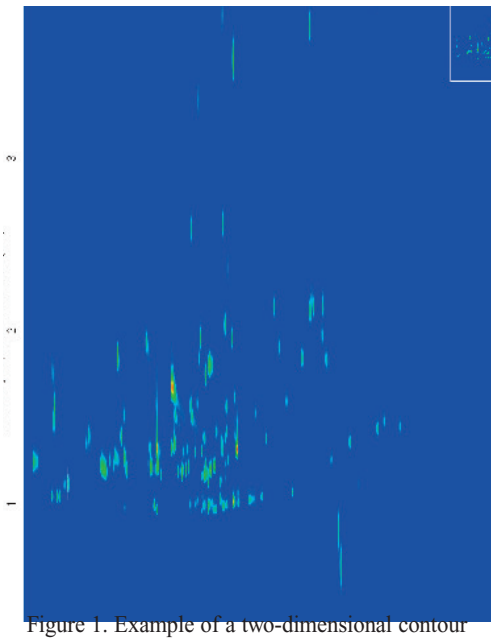

Figure 1. Example of a two-dimensional contour chromatogram generated using a GCxGC TOF-MS system. Data collected from a leaf of *Arundinaria gigantea*, a non-isoprene emitting species of bamboo.

took approximately 1 hour to prepare for BVOC sampling, and 1 hour to cycle through the GCxGC TOF-MS.

Analysis of Individual Compounds

Each sample from the GCxGC TOF-MS was analyzed using Pegasus ChromaTOF software that identifies individual peaks in the two-dimensional space, and compares the mass spectra of each peak to a NIST library compound identification system. Peaks with a signal to noise ratio lower than 200 were automatically discarded. Silicon, which is a product of SPME degradation, was deleted from all samples prior to statistical analysis. Compounds found in blanks were deleted if the Peak Area was within two to three times of that found in the blanks. The spectra of each compound was compared to the NIST library match to check for mis-identifications and to combine peaks which labeled twice or exceeded the four second modulation slice. Peak Area was used as a proxy for abundance for each compound. Peak area is based on the magnitude of the peak, however the sensitivity and response of the TOF-MS detector can vary from compound to compound, and therefore Peak Area is considered as relative abundance rather than a quantitative value.

A total of 1076 distinct compounds were emitted in at least one of the 84 samples. Because such a broad range of compounds was emitted, each compound could not be classified using authentic standards, and so NIST library identification of spectra was used. Traditionally, studies of BVOCs focus on the emission of a well-characterized, small suite of compounds (Kesselmeier and Staudt 1999). To retain the diversity and magnitude of BVOCs emitted from the bamboos, the data was compiled into groups of compounds, or compound classes. Each BVOC found in a sample was assigned to a single Compound Class based on the priority assigned to functional groups by the International Union of Pure and Applied Chemists (IUPAC). Nomenclature and the structure of each compound were used to classify compounds into one of 18 Compound Classes (Table 2). The Peak Area of individual compounds were summed according to their compound classes.

Statistical analyses

A Student's t-test was performed to test for any correlation found between the total number of compounds found in each sample to its isoprene emission level (High vs. None). Data was square root transformed to normalize residuals, and all assumptions of equal variance were met. (JMP Statistical Software; SAS institute Inc., Cary, NC, USA).

To analyze the relationships between compound classes within and across samples, multivariate statistical approaches were used. The total Peak Area of each compound class was considered to be a separate response variable for each individual leaf sample. Initially, a correlation matrix was generated to examine the relationships between different compound classes across all samples in the entire dataset. The data was visually examined and then square root transformed to normalize

Table 2. Criteria for assigning compound class to each compound. Terpenoids (Hemiterpenes, Monoterpenes, Sesquiterpenes), Halides, Nitros, and Sulfurs were assigned based on formula rather than on IUPAC nomenclature.

Compound Class	Criteria for class	Compound Class	Criteria for class
Acid	"acid" in name	Furan	"furan" in name
Alcohol	-ol suffix	Halide	Contains Cl, Br, F, I
Aldehyde	-al, -yde suffix	Hemiterpene	C_5H_8
Alkane	-ane suffix	Ketone	-one suffix
Alkene	-ene suffix	Monoterpene	$C_{10}H_{16}$
Alkyne	-yne suffix	Nitros	Contains N
Dioxy.Monoterpene	$C_{10}H_{16}O_2$	Oxy.Monoterpenes	$C_{10}H_{16}O$
Ester	"ester, -ate suffix	Sesquiterpenes	$C_{15}H_{24}$
Ether	-ide suffix	Sulfurs	Contains S

the distribution of the compound classes. The correlation matrix was used to determine whether any two compound classes were correlated with one another within the dataset.

Vegan and MASS libraries were used to run non-metric multidimensional scaling (NMDS) using metaMDS. In our ordination, each leaf was considered a separate sample and analyses were run against the entire compound class composition of the leaf. NMDS plots were created for all combined samples and for the Light treatment and Dark treatment alone. The NMDS algorithm was run 20 times for each ordination with a different starting configuration each time. The final ordination was chosen based on the configuration with the lowest stress value (badness-of-fit). NMDS ordinations were generated and analyzed in two dimensions and did not exceed a stress level of eleven. Analysis of Similarity (ANOSIM) was run on the output from the combined NMDS analysis, and on the Light NMDS and Dark NMDS plots individually. The Null Hypothesis of the ANOSIM assumes no difference between leaves of different species.

All multivariate statistical analyses were preformed on compound class data in R statistical software (http://cran.stat.ucla.edu/).

RESULTS

No significant difference was found between the number of BVOCs emitted between isoprene emitters and non-isoprene emitters (p=0.1932) (Table 3). Isoprene emission presence or absence was coded according to the relative emission rates found Tables 1 and 3.

A correlation matrix was used to determine the relationships between each pairwise grouping of compound classes collectively for all samples. Strong positive correlations were found between Alcohols and Alkenes (R=0.74), Alcohols and Aldehydes (R=0.62), and Monoterpenes and Sesquiterpenes (R=0.70). Isoprene was treated as an individual compound class so that the relationship of isoprene to other classes could be determined. No significant correlation between isoprene and any other individual compound class was found.

A NMDS plot was generated for all samples combined, regardless of light treatment (Figure 2). Each point on the plot represents a single leaf sample, and the placement of the point in the ordination is determined by its overall compound class composition, and the relationship of that class composition to each other sample. NMDS plotted for light and dark species

Table 3. Compound number and isoprene emission rate for each species. Isoprene emission measurements were taken in situ from intact leaves attached to greenhouse specimens.

Genus species 'Cultivar'	**Total number of compounds (average) n=4**	**Isoprene emission rate (nmol isoprene m^{-2} sec^{-1}) n=12**	**Isoprene Emission Level**
Phyllostachys nigra	195	10.443	High
Arundo donax	141	8.524	High
Phyllostachys aurea	150	7.477	High
Bambusa ventricosa	75	6.244	High
Bambusa ventricosa 'Kimmei'	105	5.724	High
Sasa kurilensis 'Shimofuri'	156	2.136	High
Pleioblastus chino 'Murakamianus'	176	0.876	None
Phyllostachys edulis	154	0.864	None
Arundinaria gigantea	140	0.542	None
Sasa kurilensis	117	0.418	None
Fargesia rufa	123	0.410	None
Pleioblastus chino 'Vaginatus Variegatus'	196	0.386	None
Pleioblastus chino	169	0.294	None

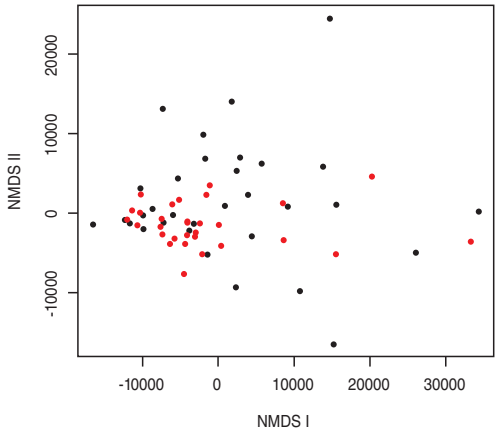

Figure 2. NMDS plot showing all bamboo (Light and Dark treatments). Each point represents a single leaf sample. Points in gray indicate samples from isoprene emitting species, points in black from non-isoprene emitting species.

separately show similar clustering of isoprene emitting and non-emitting plants.

The NMDS plot in Figure 2 was regenerated to show the abundance of sesquiterpenes emitted by each leaf (Figure 3). Each circle represents the same data point from Figure 2, and the size of the circle is directly proportionate to the amount of sesquiterpenes emitted by that sample. Points in the upper right quadrant, identified as non-isoprene emitting plants, show the highest abundance of sesquiterpene emission.

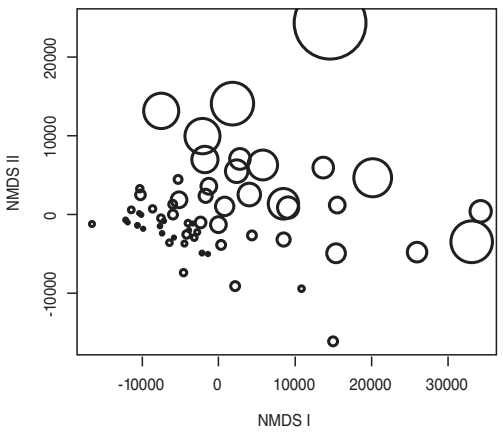

Figure 3. NMDS plot of all samples. Each point represents the same configuration seen in Fig. 2. Circles represent the relative abundance of sesquiterpenes emitted for each leaf sample.

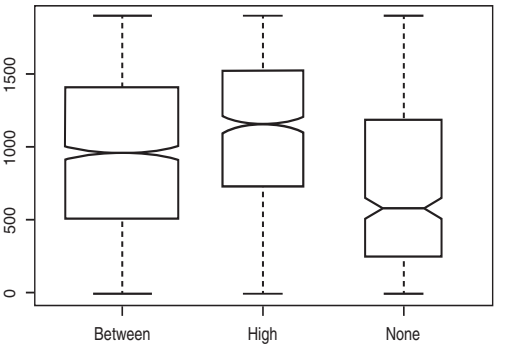

Figure 4. ANOSIM boxplot of differences in compound class composition between high and non-isoprene emitting species. “Between” shows entire range of dataset, “None” refers to non-isoprene emitting plants. N=24 “High”, N=28 “None”. R=0.035, P=0.044

An ANOSIM was generated to explore the difference in compound classes composition between isoprene emitting and non-emitting bamboo (Figure 4). The y-axis represents the distance between data points in the space, and the boxplots approximate the range of variation found within the data. The emissions of biogenic compound classes were significantly different between plants that do and do not make isoprene (R=0.035, P=0.044). Next, an ANOSIM was run to determine if compound class composition was also different amongst the thirteen species of grass surveyed in this study (Figure 5). The difference was significant (R=0.173, P=0.004) when all species were considered collectively.

DISCUSSION

Plants emit a range of volatile organic compounds, including isoprene. Generally, isoprene is conserved within plant taxa making analysis of variance and BVOC correlates with this variance difficult (Harley *et al.* 1999; Sharkey *et al.* 2008). Our data show that across bamboo species isoprene emission is variable and that the composition of other volatile organic compounds varies with isoprene emission (Figure 4). The NMDS ordination is created by examining the entire suite of BVOCs emitted across the bamboos in our study. Though no single compound class is strongly correlated with isoprene, an observable pattern of isoprene

emitters vs. non-emitters exists within the ordination space. This indicates that there is an underlying difference in the suite of BVOCs emitted from bamboos that do or do not make isoprene.

Variations in other groups of compounds are also responsible for the patterns observed in the ordination, though the ecological relationships between these compound classes are still poorly understood (Figure 3). These results are interesting because, while isoprene is by far the most abundant BVOC emitted globally, it is not considered to play a distinct role with regard to interactions in chemical ecology. Compounds such as sesquiterpenes (Kesselmeier and Staudt 1999; Duhl 2008) play crucial roles in signaling between plants, and towards pollinators and insect predators. Our results suggest that isoprene emission may come at the cost of more ecologically relevant compounds, such as sesquiterpenes (Figure 3). Plants that emitted little to no isoprene showed the highest amounts of sesquiterpenes present within their total compound class composition. These results suggest the presence of a tradeoff between leaf isoprene emission, which is often associated with heat stress, and leaf sesquiterpene emission, which is known to aide in plant defense systems (Kesselmeier and Staudt 1999). The ecological or physiological implications of this shifting pattern of BVOC emissions within the Bamboos are currently unknown.

In addition to variations in BVOCs observed across our bamboos, within a given genus the overall emission of BVOCs may vary significantly (Figure 4). Differences were observed between species within the genera *Phyllostachys* and *Pleioblastus*, indicating that a purely phylogenetic approach to understanding BVOC emissions may not be appropriate in the bamboos.

We have identified the bamboos as a novel system for understanding the complex interrelationships that may exist between the BVOCs emitted from plant leaves. Future studies will target the interactions between isoprene emissions and specific compounds, e.g sesquiterpenes. The quantity of carbon emitted by bamboos may be equally variable in addition to the compounds themselves, and while relative abundances between compound classes were variable, quantitative experiments need to be performed to elucidate these differences. We have shown here that the composition of BVOCs released in closely related bamboo species varies dramatically, providing the first glimpse of the remarkable diversity of BVOC emissions within the bamboos.

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The effect of light intensity and temperature on the chlorophyll fluorescence of *Phyllostachys aureosulcata* bamboo plants under controlled growth chamber conditions

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ABSTRACT

Chlorophyll fluorescence is often used to study the effect of environmental changes on photosynthetic performance of plant leaves. Apparently, in the evergreen bamboo genus *Phyllostachys*, when growing in the field, leaf fluorescence is subjected to seasonal and vertical variation, with a lower chlorophyll fluorescence efficiency during spring and deeper in the canopy. Therefore, it was hypothesized that the increasing light intensities from winter to spring combined with the low air temperatures in this period induce a severe but reversible stress on the operation of the photosynthetic apparatus of bamboo leaves grown in Ireland. To test this hypothesis, chlorophyll fluorescence was measured on the top leaves of *Phyllostachys aureosulcata* growing in a growth chamber under shaded and unshaded light conditions and in warm and cold air temperature. Under unshaded conditions, a reduction of Fv/Fm (the parameter most frequently used in chlorophyll fluorescence), could be observed. This reduction was even more severe under low air temperature conditions. At high air temperature, a damped oscillation or recovery could be observed after three weeks at unshaded conditions. Under shaded conditions, only a small decrease was present under high air temperature, and no decrease could be observed under low air temperature. The results of this experiment might serve as an explanation for the seasonal and vertical variation in chlorophyll fluorescence of bamboo observed in the field.

Key-words: bamboo, evergreen, Fv/Fm, light stress, photoinhibition; temperature stress.

INTRODUCTION

Bamboo is a widespread woody grass with over 1200 species in 90 genera (Crompton 2006). While not native to Europe and North America (Whittaker 2005), a number of temperate bamboo species are easily grown on these continents as if they were (Whittaker 2005; Potters *et al*. 2009; Van Goethem *et al*. 2013a). In recent years, there is a growing interest for these plants in Europe (Potters *et al*. 2013), not only for their potential as biomass producer, but also for the numerous uses as renewable bio-based materials such as wood, composites, fibres, chemicals and energy (El Bassam 1998; Van Acker *et al*. 2000; Papadopoulos *et al*. 2004; Van Hoyweghen *et al*. 2010) and for their performance in the removal of soil pollutants such as heavy metals (Potters *et al*. 2009). The bamboo genera most suitable in Europe are temperate bamboos such as *Sasa, Fargesia* and *Phyllostachys*. Among these, *Phyllostachys* species are known best for their very high biomass potential (El Bassam 1998), and can withstand air temperatures as low as -25°C (Whittaker 2005).

As plant growth and development is closely related to the physiological process of photosynthesis (Goldschmidt 1999; Vu 1999), it is important to understand how the environment

affects this latter parameter. The photosynthetic process is driven by light absorbed by the photosynthetic apparatus (Fig. 1) (Stirbet and Govindjee 2012). From the absorbed light energy (ABS), part will be dissipated in the form of heat or fluorescence (DIo), while the rest will be transported (ETo) to the reaction centre of PSII. The reaction centre (RC) (P680) and pheophytin (Pheo) function as primary donor and acceptor molecules of PSII respectively. The 'energy trapping' (TRo) process is completed when the separated charges are 'stabilized' by electron transfer from reduced Pheo to the primary quinine acceptor (Q_A) on the acceptor side, and from the electron donor (YZ) to oxidized P680 on the donor side (Kramer *et al*. 2004; Schreiber 2004).

As the intensity of the chlorophyll fluorescence is modulated by the redox state of Q_A as well as some other redox components of PSII, the variable yield of chlorophyll fluorescence provides a useful diagnostic tool for studying electron transport (Tyystjärvi & Vass 2004). Fluorescence spectroscopy has been used to study the regulation of photosynthesis in leaves (Ribeiro *et al*. 2009) and to assess the possible damages to the photosynthetic apparatus due to different forms of stress (Papageaorgiou & Govindjee 2005), such as light intensity (Tsimilli-Michael, Krüger, & Strasser 1995; Srivastava & Strasser 1996; Krüger, Tsimilli-Michael, & Strasser 1997), air temperature (Guissé, Srivastava, & Strasser 1995; Srivastava *et al*. 1997; Strasser 1997), soil drought (Van Rensberg *et al*. 1996) or chemical influences, such as copper (Ouzounidou 1997).

One of the most frequently used parameters in chlorophyll fluorescence is the ratio between variable and maximum fluorescence (Fv/Fm). This parameter denotes the potential quantum efficiency of PSII (Krause & Jahns 2004). Across a wide range of higher plants species, this parameter has an optimal value of 0.83 (Demmig-Adams *et al*. 1996). When exposed to abiotic and biotic stresses, the Fv/Fm in plants will decrease (Baker 2008). Therefore, this parameter is frequently used as a stress detector under environmental stress conditions (Öquist & Huner 1991; Lichtenthaler & Brukart 1999; Ogaya *et al*. 2011).

To study the structural and functional behavior of PSII, other parameters of chlorophyll a fluorescence can also be used to analyse the efficiency in the capturing and use of light energy (Table 1):

- Fo, Fm, Fv/Fo (=(Fm-Fo)/Fo) (as these parameters characterize fluorescence induction (Bolhar-Nordenkampf et al. 1989)),
- the specific fluxes expressed per reaction center (since the efficiency of the electron transport chain is related to the efficiency of photon absorption and the subsequent transport and trapping of the electrons), the maximum quantum efficiency of PSII primary photochemistry (TRo/ABS), the maximum efficiency of non-photochemical de-excitation (DIo/ABS),

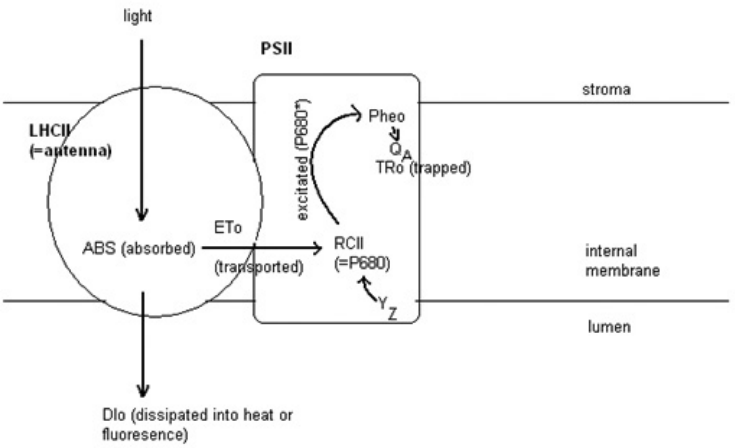

Figure 1: Schematic representation of the first part of the electron transport. [adapted from (Kramer et al. 2004; Schreiber 2004)]

Table 1: Used chlorophyll fluorescence parameters and their definitions, for further details, see Strasser *et al.* (2004), Gonçalves et al. (2007) and Kalaji *et al.* (2011) . PSII= Photosystem II, RC= reaction centre, QA=quinone A

Parameters	**Definitions**
Fo	Initial fluorescence, when all PSII RCs are open
Fm	Maximum fluorescence
Fv (=Fm-Fo)	Variable fluorescence
Fv/Fo (=(Fm-Fo)/Fo)	The efficiency of the water-splitting complex on the donor side of PSII
Specific fluxes (RC)	
ABS/RC	Effective antenna size of an active RC
ETo/RC	Electron transport of an active RC
Tro/RC	Maximal trapping rate per RC
DIo/RC	Dissipation of an active RC
Yields	
TRo/ABS (=Fv/Fm)	Maximum quantum yield of primary photochemistry
DIo/ABS (=Fo/Fm)	Maximum quantum yield of non-photochemical de-excitation
ETo/TRo	Probability that a trapped exciton moves an electron further than Q_A
ETo/ABS	Probability that an abosrbed photon moves an electron further than Q_A
Performance index	
PI_{abs}	Performance index

and the probability that a trapped exciton (ET/TRo) or that an absorbed photon (ETo/ABS) moves an electron further than quinone A (Q_A),

- the performance index PI_{abs} defined as:

$$PI_{abs} = \left(\frac{RC}{ABS}\right)\left(\frac{Fv/Fm}{1-Fv/Fm}\right)\left(\frac{ETo/TRo}{1-ETo/TRo}\right)$$

This last parameter combines three independent functional steps of photosynthesis, such as the density of reaction centers in the chlorophyll bed, excitation energy trapping and conversion of excitation energy to electron transport, into a single multi-parametric expression (Srivastava & Strasser 1999; Strasser, Srivastava, & Tsimilli-Michael 2000). PI_{abs} allows broader analysis of photosynthetic performance and is therefore a better parameter to evaluate responses of PSII to stressful conditions than Fv/Fm alone (Gonçalves *et al*. 2007).

In the field, seasonal and diurnal dynamics in chlorophyll fluorescence have been reported for different plant species such as Mediterranean shrubs and grassland species (Fernandez-Baco *et al*. 1998; Ain-Lhout *et al*. 2004; Zunzunegui *et al*. 2010), conifers (Adams *et al*. 2002; Nippert, Duursma, & Marshall 2004), and for bamboo species (Van Goethem *et al*. submitted; Kumar *et al*. 2002). Since photon flux densities can vary over different seasons, days and even shorter periods (Goodale, Aber, & Ollinger 1998) and over a wide range (2500µmol $m^{-2}s^{-1}$ for sun plants, while shade plants often receive below 100 µmol $m^{-2}s^{-1}$), it is suggested that light intensity might induce these dynamics in chlorophyll fluorescence. In the literature, light stress is often referred to as an important stress factor that can induce photoinhibition (Demmig-Adams & Adams 1992; Long & Hymphries 1994; Lichtenthaler & Brukart 1999). If the amount of absorbed light exceeds the ability of photochemical use in the photosynthetic apparatus, the surplus energy has to be rendered harmless by the photosynthetic apparatus via deactivation mechanisms (Baker & Horton 1987). If leaves are subjected to high light intensities and low air temperatures (photochilling), a pronounced photoinhibitory decrease of Fv/Fm can often be observed (Bongi & Long 1987; Bolhar-Nordenkampf & Lechner 1988; Lechner & Bolhar-Nordenkampf 1989; Baker *et al*. 1989).

Table 2: Mean values of the fluorescence parameters Fo,Fm,Fv/Fo, Fv/Fm, the specific fluxes (ABS/RC, TRo/RC, ETo/RC and DIo/RC), the yields (DIo/ABS,ETo/ABS and ETo/TRo) and performance index (PI_{abs}) for every week at high light and high air temperature conditions (L_HT_H). Different letters denote significant (P<0.01) differences between weeks (Tukey).

time (weeks)	1	2	3	4	5
Fo	773.67^{a,b}	867.18^{a}	882.10^{c}	753.42^{b}	714.39^{b}
Fm	2508.10^{a}	2128.77^{b}	1996.79^{b,c}	2180.10^{b}	1878.78^{c}
Fv/Fo	2.29^{a}	1.53^{b}	1.32^{b}	1.95^{a,c}	1.66^{b,c}
Fv/Fm	0.68^{a}	0.56^{b}	0.53^{b}	0.63^{a}	0.58^{b}
ABS/RC	0.93^{a}	1.09^{a,b}	1.24^{b}	1.05^{a,b}	1.42^{a,b}
TRo/RC	0.62^{a}	0.57^{a}	0.61^{a}	0.65^{a}	0.70^{a}
ETo/RC	0.52^{a}	0.41^{a}	0.43^{a}	0.55^{a}	0.55^{a}
DIo/RC	0.32^{a}	0.51^{a}	0.64^{a}	0.41^{a}	7.10^{a}
DIo/ABS	0.32^{a}	0.44^{a}	0.47^{a}	0.37^{a}	0.42^{a}
ETo/ABS	0.57^{a}	0.43b,c	0.38^{c}	0.53^{a,d}	0.48^{b,d}
ETo/Tro	0.83^{a}	0.73^{b}	0.70^{b}	0.84^{a}	0.80^{a}
PI_{abs}	304.30^{a}	143.45^{a,b}	83.86^{b}	238.07^{a,b}	168.61^{a,b}

In the bamboo species *Phyllostachys humilis*, seasonal and vertical variations in chlorophyll fluorescence have been related to the levels of light, air temperature and precipitation in field conditions (Van Goethem *et al*. 2013b). Despite this relation, it has not been verified whether changing levels of light and temperature indeed induce these dynamics in chlorophyll fluorescence in bamboo. In order to test this hypothesis, the effect of light and air temperature was assessed on the onset of photoinhibition in *Phyllostachys aureosulcata* and on the subsequent potential recovery in the leaves under controlled conditions.

MATERIALS AND METHODS

Phyllostachys aureosulcata of 3 years old, produced via in vitro techniques and delivered by Oprins Plant NV (Rijkevorsel, Belgium) in a 10 L pot, were placed into a growth chamber for 4 weeks. The average height of the plants was about 1 m. To test the effect of light and air temperature on leaf chlorophyll fluorescence, the plants were divided in 4 trials:

1) warm conditions without shading (L_HT_H, Light High and Temperature High).
2) warm conditions with shading (L_LT_H, Light Low and Temperature High).

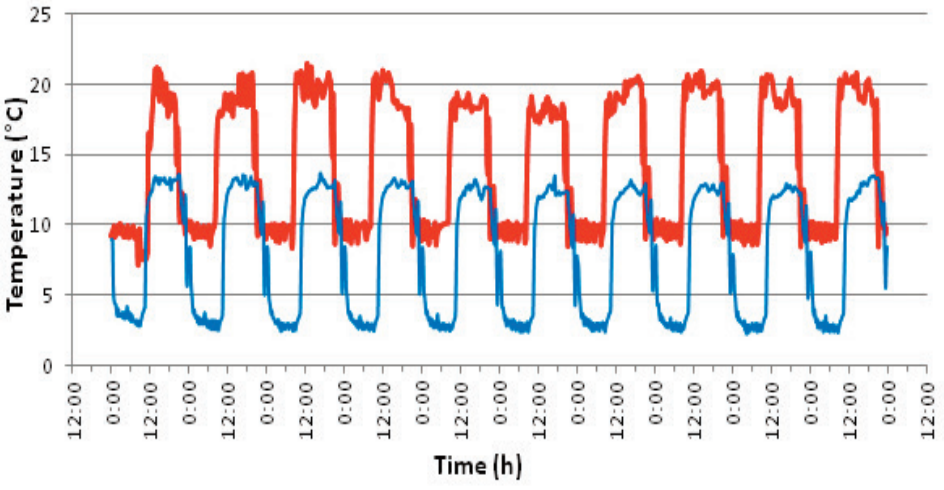

Figure 2: Air temperature fluctuation of the growth chamber. The upper line represents the warm conditions, the lower line represents the cold condition.

Table 3: Mean values of the fluorescence parameters Fo,Fm,Fv/Fo, Fv/Fm, the specific fluxes (ABS/RC, TRo/RC, ETo/RC and DIo/RC), the yields (DIo/ABS,ETo/ABS and ETo/TRo) and performance index (PI_{abs}) for every week at high light and low air temperature conditions (L_HT_L). Different letters denote significant (P<0.01) differences between weeks (Tukey).

time (weeks)	1	2	3	4	5
Fo	717.02^{a}	732.66^{a}	655.33^{b}	689.33^{a,b}	657.00^{b}
Fm	2238.25^{a}	1396.15^{b}	1111.17^{c}	1159.05^{c}	1042.04^{c}
Fv/Fo	2.15^{a}	0.89^{b}	0.67^{b,c}	0.65^{b,c}	0.56^{c}
Fv/Fm	0.68^{a}	0.43^{a,b}	0.36^{b}	0.36^{a,b}	0.34^{c}
ABS/RC	0.84^{a}	2.15^{a}	8.46^{a}	8.10^{a}	6.20^{a}
TRo/RC	0.67^{a}	0.77^{a}	1.87^{a}	1.95^{a}	1.29^{a}
ETo/RC	0.58^{a}	0.57^{a}	1.49^{a}	1.67^{a}	0.89^{a}
DIo/RC	0.18^{a}	1.38^{a}	6.59^{a}	6.14^{a}	4.92^{a}
DIo/ABS	0.32^{a}	0.57^{b}	0.64^{c}	0.64^{c}	0.66^{c}
ETo/ABS	0.59^{a}	0.33^{b}	0.28^{b,c}	0.28^{b,c}	0.25^{c}
ETo/Tro	0.87^{a}	0.75^{b}	0.75^{b}	0.75^{b}	0.72^{b}
PI_{abs}	510.43^{a}	79.18^{b}	27.35^{b}	34.77^{b}	22.19^{b}

3) cold conditions without shading (L_HT_L, Light High and Temperature Low).
4) cold conditions with shading (L_LT_L, Light Low and Temperature Low).

Light intensity was measured with a Serial Quantum Sensor (Hansatech Instruments Ltd.). Unshaded plants (L_H) received a light intensity of about 370 µmol $m^{-2}s^{-1}$, whereas the plants protected by a shade cloth (L_L) received only about 80 µmol $m^{-2}s^{-1}$ (both measured at the top of the plants). Although 370 µmol $m^{-2}s^{-1}$ is not as high as the light intensities measured in full sunlight in Ireland (up to 2000 µmol $m^{-2}s^{-1}$), this was the highest light intensity that could be reached in the growth chamber with artificial light. The top of the plants was ±1 m distant from the lamps. Air temperature was controlled by an airconditioning system and there was a difference of 7°C between warm (T_H) and cold (T_L) conditions. Plants were maintained under a 12-h photoperiod with 60% relative air humidity during the dark regime and 40% relative air humidity during the light regime, and temperature was kept at 3°C and 10°C during

Table 4: Mean values of the fluorescence parameters Fo,Fm,Fv/Fo, Fv/Fm, the specific fluxes (ABS/RC, TRo/RC, ETo/RC and DIo/RC), the yields (DIo/ABS,ETo/ABS and ETo/TRo) and performance index (PI_{abs}) for every week at low light and high air temperature conditions (L_LT_H). Different letters denote significant (P<0.01) differences between weeks (Tukey).

time (weeks)	1	2	3	4	5
Fo	705.85^{a}	686.46^{a,b}	667.51^{a,b}	644.82^{b}	662.65^{a,b}
Fm	2888.49^{a}	3253.76^{b}	3246.56^{b}	3063.03^{a,b}	2963.61^{a}
Fv/Fo	3.11^{a}	3.75^{b,c}	3.88^{b}	3.74^{b,c}	3.46^{c}
Fv/Fm	0.75^{a}	0.79^{b}	0.79^{c}	0.79^{c}	0.77^{c}
ABS/RC	0.86^{a}	0.75^{a}	0.84^{a}	0.80^{a}	0.74^{a}
TRo/RC	0.63^{a}	0.59^{a}	0.66^{a}	0.63^{a}	0.57^{a}
ETo/RC	0.57^{a}	0.55^{a}	0.62^{a}	0.59^{a}	0.53^{a}
DIo/RC	0.23^{a}	0.16^{a}	0.18^{a}	0.17^{a}	0.17^{a}
DIo/ABS	0.25^{a}	0.21^{b,c}	0.21^{b}	0.21^{b,c}	0.23^{c}
ETo/ABS	0.67^{a}	0.73^{b}	0.74^{b}	0.74^{b}	0.72^{b}
ETo/Tro	0.90^{a}	0.93^{b}	0.93^{b}	0.93^{b}	0.93^{b}
PI_{abs}	786.12^{a}	818.52^{a}	1304.25^{a}	1146.94^{a}	1332.75^{a}

Table 5: Mean values of the fluorescence parameters Fo,Fm,Fv/Fo, Fv/Fm, the specific fluxes (ABS/RC, TRo/RC, ETo/RC and DIo/RC), the yields (DIo/ABS,ETo/ABS and ETo/TRo) and performance index (PI_{abs}) for every week at low light and low air temperature conditions (L_LT_L). Different letters denote significant (P<0.01) differences between weeks (Tukey).

time (weeks)	1	2	3	4	5
Fo	705.85^{a}	686.46^{a,b}	667.51^{a,b}	644.82^{b}	662.65^{a,b}
Fm	2888.49^{a}	3253.76^{b}	3246.56^{b}	3063.03^{a,b}	2963.61^{a}
Fv/Fo	3.11^{a}	3.75^{b,c}	3.88^{b}	3.74^{b,c}	3.46^{c}
Fv/Fm	0.75^{a}	0.79^{b}	0.79^{c}	0.79^{c}	0.77^{c}
ABS/RC	0.86^{a}	0.75^{a}	0.84^{a}	0.80^{a}	0.74^{a}
TRo/RC	0.63^{a}	0.59^{a}	0.66^{a}	0.63^{a}	0.57^{a}
ETo/RC	0.57^{a}	0.55^{a}	0.62^{a}	0.59^{a}	0.53^{a}
DIo/RC	0.23^{a}	0.16^{a}	0.18^{a}	0.17^{a}	0.17^{a}
DIo/ABS	0.25^{a}	0.21^{b,c}	0.21^{b}	0.21^{b,c}	0.23^{c}
ETo/ABS	0.67^{a}	0.73^{b}	0.74^{b}	0.74^{b}	0.72^{b}
ETo/Tro	0.90^{a}	0.93^{b}	0.93^{b}	0.93^{b}	0.93^{b}
PI_{abs}	786.12^{a}	818.52^{a}	1304.25^{a}	1146.94^{a}	1332.75^{a}

the dark regime, and 13°C and 20°C during the light regime for warm and cold conditions, respectively (Fig. 2). In this way, cold and warm conditions were comparable with, respectively, spring (March – April) and summer (July-August) values in Ireland (Goodale *et al.* 1998). Plants were weekly watered with tap water to avoid water stress. Air temperature and relative humidity were recorded at plant height using a Helios Logger (Skye Instruments Ltd.).

Ten plants were followed up per treatment. On one culm of each plant, six leaves of the top two nodes were used. Fluorescence measurements were taken in the middle of the leaf between 1pm and 5pm with the Handy Pea (Hansatech instruments Ltd., England, Norfolk) after dark adaptation with leaf clips for 30 minutes. Changes in the photochemical capacity of PSII were studied by calculating the fluorescence parameters described in Table 1 with the Biolyzer HP 3.0 (Fluoromatics Software).

All the leaves were weekly measured, and the start of the experiment was taken as week 1. Data were analysed in the statistical program

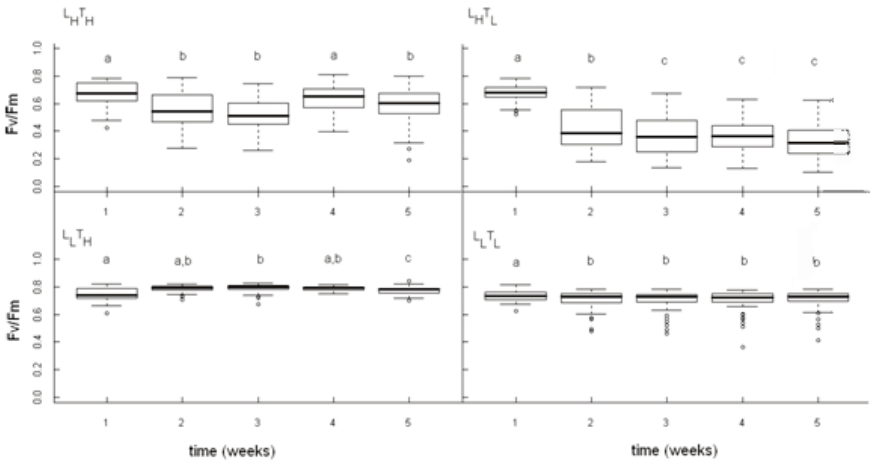

Figure 3: Fv/Fm values in function of time (weeks) at high light and high air temperature conditions (L_HT_H), high light and low air temperature conditions (L_HT_L), low light and high air temperature conditions (L_LT_H) and low light and low air temperature conditions (L_LT_L). Different letters denote significant (P<0.01) differences between weeks (Tukey)

R v2.13.0 (R development Core Team 2011) using mixed models with leaf nested in plant (Pinheiro *et al*. 2011). Significant differences were tested using Tukey's all-pairwise comparisons at 1% probability.

RESULTS

Under unshaded conditions (L_H), a statistically significant decrease in the parameters Fm, Fv/Fo, Fv/Fm, ETo/TRo, ETo/ABS and PI_{abs} and an increase in DIo/ABS and Fo could be observed under low air temperature (T_L) as well as under high air temperature (T_H) (Table 2,3). This effect was already present after one week at both air temperatures, and even more severe at T_L (Table 3). After three weeks, an opposite change in these parameters could be observed at T_H (Table 2), whereas at T_L, the Fv/Fm values decreased even further (Table 3).

Under shaded conditions (L_L) no statistically significant decrease was present in the PI_{abs}values (Table 4,5). At L_LT_H, although to a limited extent, significant increases and decreases could be observed in Fm, Fv/Fo and Fv/Fm and an antiphase pattern in the DIo/ABS values. Also a small decrease in Fo and increase in ETo/ABS and ETo/TRo were present in the first week under these conditions (Table 4).

At L_LT_L there were neither changes in Fo nor in ETo/TRo and only a small decrease is shown in the parameters Fm, Fv/Fo, Fv/Fm and ETo/ABS with DIo/ABS showing a simultaneous increase (Table 5).

Between the different light conditions, we could observe a large difference in variances in Fv/Fm values, with a larger variation in plants under L_H compared to those under L_L, as the relative standard deviation of Fv/Fm was 24% and 38% at L_HT_H and L_HT_L and 5% and 1% at L_LT_H and L_LT_L, respectively, in the last week of the experiment (Fig. 3).

In all conditions, no significant changes could be observed in ETo/RC,DIo/RC, TRo/RC and only small but significant changes in L_HT_H in ABS/RC (Table 2,3,4 and 5).

DISCUSSION

In both warm and cold air temperature conditions, the Fv/Fm values of *P. aureosulcata* are reduced when their leaves are placed under light intensities of 370 µmol $m^{-2}s^{-1}$, whereas only small changes were observed under light intensities of 80 µmol $m^{-2}s^{-1}$. Although 370 µmol $m^{-2}s^{-1}$ is not an extreme high value, as ambient photo flux densities can reach up to 2500 µmol $m^{-2}s^{-1}$, apparently, this value can cause a decrease in photochemical efficiency in the leaves, probably as a stress signal. A light-induced decline of photosynthetic activity is broadly termed as photoinhibition (Vass 2011). Although photoinhibition is mostly viewed as a process of stress-induced damage to photosystem II (Kyle *et al*. 1984; Prasil, Adir, & Ohad 1992; Rintamaki *et al*. 1995), it can also be considered as a protective mechanism against high light, since photoinhibition results from the formation of photochemically inactive photosystem II centers, which convert the excitation energy into heat (Cleland, Melis, & Neale 1986; Aro, Virgin, & Andersson 1993; Gilmore & Bjorkman 1994).

The variation in Fv/Fm can be interpreted by the separation of the effects of non-photochemical quenching, by analyzing the individual parameters Fo, Fm and Fv/Fo (Long & Hymphries 1994). If PSII is not functional, the decline in Fv/Fm would be explained by an increase in Fo (Krause & Jahns 2004). If xanthophyll quenching and heat-deactivating centers are responsible, a drop in Fm would decrease Fv/Fm, although there may also be a drop in Fo. The Fv/Fo ratio reflects the efficiency of electron donation to the PSII reaction center and the rate of photosynthetic quantum conversion at the PSII reaction center (Lichtenthaler & Babani 2004). A decrease in Fv/Fm values can be due to an increase in Fo and/or a decrease in Fm. An increase in Fo values can be caused by a partially closed PSII reaction center or a malfunction in the transfer of the excitons from the antenna to the reaction center (Guidi *et al*. 2006). When the excitons cannot reach the reaction center, they will fall back to their ground state, evoking a rise in high fluorescence. On the other hand, a decrease in Fm values can be a consequence of an increased loss of energy by heat (instead of fluorescence), suggesting that xantophyll quenching and heat-deactivating centers are responsible and perhaps an enhancement of light distribution to PSI (Bolhar-Nordenkampf, Hofer, & Lechner

1991; Krause & Weis 1991; Figueroa *et al*. 1997). In both unshaded conditions, the decline in Fv/Fm coincides with a reduction in Fm, consistent with non-photochemical quenching (Table 1,3). Only in unshaded conditions under high air temperature, this coincides with a small but significant increase in Fo. The loss of energy in the form of heat is confirmed by the antiphase oscillation pattern in L_HT_H and the rising values in L_HT_L of DIo/ABS and DIo/RC. Moreover, the decline in Fv/Fo indicates structural alterations between the components in PSII, as not all excitons will reach the reaction center and fluorescence will rise (Lichtenthaler & Babani 2004). Some researchers (Stroch *et al*. 2004; Kim *et al*. 2009) suggest that photoinhibition depends on the antenna size of PSII, while others (Tyystjärvi & Vass 2004) claim that it does not depend on the LHCII size (Apostolova 2012). We found that the values of ETo/RC, TRo/RC and ABS/RC do not show any significant differences. Therefore, we can conclude that there is no change in effective antenna size and the maximal trapping rate of PSII. However, there is an antiphase oscillation pattern and a decrease in ETo/TRo and ETo/ABS in L_HT_H and L_HT_L, respectively, indicating a loss in efficiency in the first part of the electron transport, from the reaction centers to Q_A.

The rise in Fv/Fm and PI_{abs} values after three weeks under unchanged environmental conditions at L_HT_H (Table 2) could be due to a damped oscillation or a recovery, suggesting that the plants are adapting to their environment and that they re-activate these inactive PSII centers. On the contrary, in L_HT_H, Fv/Fm values decreased even under the value of 0.4, indicating irreversible stress (Table 3).

The re-activation observed under L_HT_H could be an adaptive mechanism of *P. aureosulcata* to protect PSII from damage. As a consequence, an adaptive mechanism can also explain the large variability in Fv/Fm values of the leaves in the unshaded conditions compared with the rather limited variability in Fv/Fm at shaded conditions (Fig. 3), as not every leaf will form (as quickly) the same number of inactive PSII centers, even under similar conditions. Lazár *et al*. (2005) previously have shown that stress factors can increase the variance of different chlorophyll fluorescence parameters.

Under low light levels, less variations could be observed, and the observed variations were, although significant, to a more limited extend. Under L_LT_H (Table 4), Fv/Fm values do not drop under their initial values, suggesting that these plants were not stressed under these conditions. Under L_LT_L (Table 5), a small decline in Fv/Fm values was present, compared to the large decline in the leaves of the plants at higher air temperature, consistent with the excitation pressure hypothesis (Huner *et al*. 1996; Huner, Öquist, & Sarhan 1998). This hypothesis states that at low air temperatures, photoinhibition may already occur under quite low light levels, as plants grown at 5°C and 250 µmol $m^{-2}s^{-1}$ exhibit PSII excitation pressures comparable or slightly higher than that of plants grown at 20°C but 800 µmol $m^{-2}s^{-1}$ (Huner *et al*. 1996, 1998). At unshaded conditions, a decrease in photochemical efficiency could be observed in the first week in both high and low air temperature. The extent of the effect, in agreement with Huner *et al*. (1996), is largest under low air temperature. (Table 3,5).

The light and air temperature conditions under L_HT_L are comparable with spring conditions in Ireland. In this season, a decrease and larger variation in Fv/Fm values was observed in the field for *P. humilis* in Ireland (Van Goethem *et al*. 2013b). Photochemical efficiency in the leaves on the top of the canopy was also lower than in the lower leaves (Van Goethem *et al*. 2013b), which might be explained by the fact that the top leaves receive higher light intensities and are less protected from low air temperature conditions (Oliveira & Peñuelas 2000) compared to the leaves deeper in the canopy. Just as in the results presented here, these described spring conditions are expected to induce photoinhibition in the top leaves.

P. humilis and *P. aureosulcata* are both hardy bamboo species with a hardiness of -25 °C. They originate from central China and can grow in subtropical to temperate climates (Whittaker 2005). Therefore, we can expect these species to respond similarly on the here imposed environmental stresses. Also, a similar recovery of Fv/Fm under the high air temperature treatment (under unshaded conditions) in *P. aureosulcata*, could also be observed in

P. humilis under natural conditions in Ireland, as the Fv/Fm values were recovering in early summer, when air temperature levels were rising, even though the stressing factor (light) was still present and even increasing (Van Goethem *et al*. 2013b). Even though a relation between a low precipitation and decreasing Fv/Fm values was found in Ireland (Van Goethem *et al*. 2013b), this study shows that an effect on Fv/Fm can also be expected under well-watered conditions.

CONCLUSIONS

In field conditions, *Phyllostachys* spp. showed seasonal and vertical variations in chlorophyll fluorescence. This research shows that light and air temperature are environmental parameters that can induce changes in the chlorophyll fluorescence parameters of the leaves, which has not been examined before in bamboo spp. Even though under the controlled conditions, light intensities were not extremely high (less than 500 µmol $m^{-2}s^{-1}$), and air temperature was not extremely low (above freezing temperatures (> 0°C)), the leaves of *P. aureosulcata* showed changes in the fluorescence parameters that characterize fluorescence induction. Lower Fv/Fm values, due to a decrease in Fm, and lower Fv/Fo values in leaves under unshaded conditions, suggest that an adaptive mechanism of *P. aureosulcata* is protecting PSII from damage, by performing structural alterations in PSII. The transport rates (ETo/TRo and ETo/ABS) indicate a loss in efficiency in the first part of the electron transport to Q_A and the loss in energy in the form of heat was reflected in the dissipation rate and dissipation yield (DIo/RC and DIo/ABS). Fv/Fm shows itself as a good general parameter for light and temperature stress. However, for more detailed interpretation of the involved reaction mechanisms, specific fluxes and yields should be considered. PI_{abs} in these cases seem not to be a good indicator of the stress factors mentioned above.

The same adaptive mechanism might be responsible for the protection of the PSII of these plants under a combination of low air temperatures and relatively high light intensities, as e.g. observed in spring time in temperate regions. Based on these results, both species might be able to adapt their photosynthetic system at various environments, making them potential biomass producers worldwide.

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Bamboo Mat Making and its Contribution to the Rural Livelihood of Women in South Meghalaya, India

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ABSTRACT

Over three decades, studies worldwide have witnessed the important contribution of non-timber forest products towards rural livelihoods. Forests of Meghalaya are richly endowed with bamboo species which are put to varied uses by local tribal communities. Bamboo plays a very significant role in rural livelihood by providing employment and cash income to local people through crafts and production of artifacts. The present study was conducted in the southern parts of the East Khasi Hills, Meghalaya using standard ecological, sociological and ethnographical research methods involving 120 households. The study revealed that a large percentage of the households in the study area are involved in bamboo craft. Bamboo mat making is considered as one of the important occupations and supplements the income of the rural people especially women during periods of the year where employment is scarce . The people of the area have also evolved traditional bamboo management practices that ensure continuous supply of bamboo as well as contribute towards conservation of bamboo resources of the region.

Keywords: Bamboo, Traditional craft making, Income, Gender, Seasonality, Marketing

INTRODUCTION

The role and importance of non timber forest products (NTFPs) in daily lives of the rural people in developing countries cannot be overlooked. For more than three decades, numerous studies have been conducted on potential role of NTFPs as safety nets for the rural poor and their contribution towards enhancing rural livelihood whilst simultaneously conserving forests (Ambrose-Oji 2003, Mahapatra *et al.* 2005, Tiwari 2005, Delang 2006). The sale of NTFPs and products manufactured out of these, form a part of a livelihood diversification strategy of forest dependent poor (Vormisto 2002, Shakleton and Shakleton 2004). Further, sale of NTFPs assist weaker section of society (women) to cope with adversity (Shackleton and Campbell 2007).

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In tropics and sub-tropics, bamboo constitutes a major component of NTFPs. Tribal communities use this NTFP for a variety of purposes ranging from construction and craft to food, fodder and medicine (Marden and Brandenburg 1980, Bhatt *et al.* 2003). Bamboo also plays a significant role in sustaining the livelihood of many communities residing near forest fringes (Sosola-Banda and Johnsen 2005, Bhattacharya 2007).

Bamboo belongs to the subfamily Bambusoideae of the family Poaceae and comprises 1,575 species of woody and herbaceous bamboos (Ohrnberger 1999). Over 70 genera of bamboo species cover an area of 14 million ha worldwide (Dransfield and Widjaja 1995). About 80 per cent of the species are confined to China, India and Myanmar and are found in 7 million ha of bamboo forests (Zhengyi *et al.* 2006 and Newman *et al.* 2007). In Meghalaya, bamboo forest covers an area of 4,793 sq km

(FSI 2011). Thirty five bamboo species, under 11 genera have been recorded from Meghalaya (Kharlyngdoh and Barik 2008) and the most commonly used species are: *Bambusa balcooa, B. bambos, B. cacharensis, B. jaintiana, B. nutans, B. tulda, Dendrocalamus hamiltonii, D. hookeri, D. sikkimensis, D. strictus, Melocanna baccifera, Schizostachyum dullooa* and *S. mannii*. These bamboo species are used by a large population of rural Meghalaya as the principal construction material for building houses, for making mats, baskets, and handicrafts and as food (Bhatt *et al.* 2003, Kharlyngdoh and Barik 2008).

Bamboo contributes significantly to the state government and district council's exchequers by way of collection of royalty. The average annual production of bamboo in Meghalaya is 38,568 metric tonne (Tiwari and Kumar 2008). A total of 442 tonnes of fresh bamboo shoots and 39.2 tonnes of fermented bamboo shoots are sold at the market places of Meghalaya (Bhatt *et al.* 2005). There are 495 units of bamboo and cane based handicraft in the state, providing livelihoods to 1820 persons (Anonymous 2011).

Although a good number of researches are available on NTFPs in general, the contribution of bamboo to rural economy has not received due attention. This paper aims at analyzing the role of bamboo mat making in the livelihood of rural poor of south Meghalaya and brings to fore how these communities are conserving this vital biological resource.

MATERIALS AND METHODS

Study area

The study was conducted in the southern parts of East Khasi Hill District, Meghalaya, where people are traditionally involved in plantation, management, harvesting of bamboo, and processing and marketing of bamboo products. Four villages viz., Tangmang, Korblang, Mawdang and Nongskhen were selected for the study (Fig. 1 & Table 1). The area is characterized by rugged terrains and steep slopes. The natural vegetation mainly consists of of sub tropical moist evergreen forests which are very rich in biodiversity (Tynsong 2011). It is situated at an altitude of 800-1100 m and experiences very high rainfall which ranges from 600 cm to 1000 cm annually. The inhabitants are mainly tribal people belonging to the sub-tribe War Khasi. The topography of the area is such that it is not conducive for any kind of settled agriculture. For most part of the year the people are engaged in agroforestry related activities. Agro-forestry system is mainly in the form of forest and home garden. A number of NTFPs (bay leaf, broomgrass and wild pepper) are cultivated in their forest gardens and these generate a considerable cash income (Tynsong and Tiwari 2010). People also largely depend on cultivation of cash crops like betel nut, betel leaf and oranges for sustaining their livelihood. The majority of the population, who do not own land, works as agricultural labourers in others' land. Apart from these activities, few are government employees with secure incomes. People of the area possess special skills in bamboo and cane craft making.

Data collection

The field work for collecting village and household level data was conducted between October, 2010 and November, 2011. Informal discussions were held with the headman and a group of knowledgeable persons of each village, ranging from middle to old aged persons, to know about the management systems, the extent of

Table 1 Socio-economic characteristic of the study villages

Village	Area (ha)	Population (Nos.)	Households (Nos.)	Main occupation
Tangmang	619	1700	360	Agro-forestry activities and Daily wage labourer
Korblang	425	450	80	Agro-forestry activities and Daily wage labourer
Mawdang	492	1100	200	Trade & Agro-forestry activities
Nongskhen	785	2200	372	Trade and Agro-forestry activities

Source: Community Development Block, Pynursla, 2010

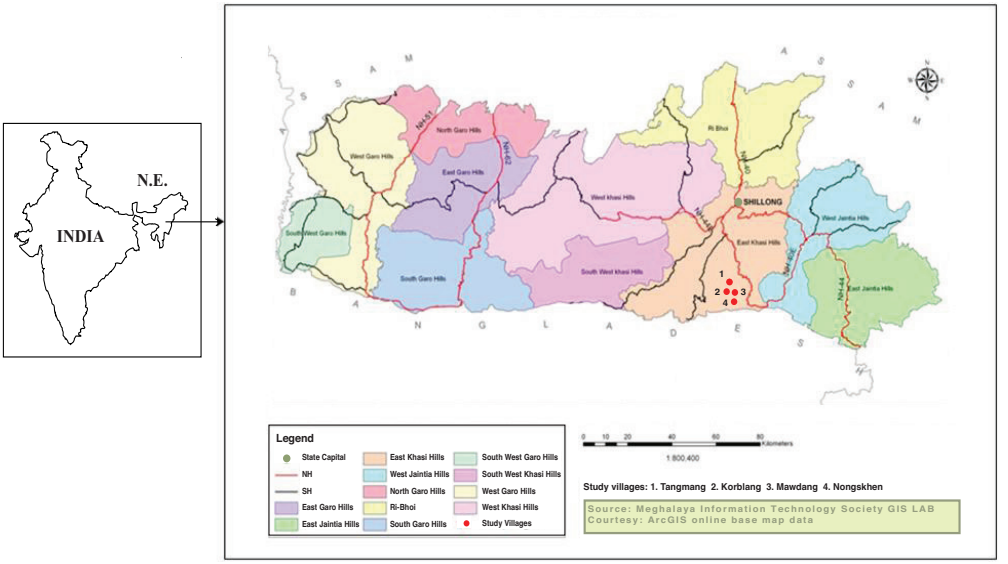

Fig 1. Location map of the study area showing the study villages

the availability of bamboos, the traditional knowledge about bamboo crafts and marketing channels of the bamboo products. PRA exercise was conducted to draw the bamboo resource map of the area. Household level survey was conducted with the help of a household questionnaire Thirty households were randomly selected from each village for the survey. Data on income obtained from mat-making, quantity of mat produced per year and gender involved in mat making were collected. Local markets were surveyed to find out the trade of these mats.

RESULTS

Mat making process

Craft making can be considered as a kind of traditional occupation which had been going on for generations. Generally women are proficient in weaving mats and males make baskets, caskets and stools. The traditional methods of mat making processes are as follows:

- *Bambusa jaintiana* locally called as 'shken', and *Calamus floribundus*, locally called as 'thri' are used as raw materials for mat making.
- About 20-30 straight, well formed bamboos of 2 meter length and 5 cm diameter and with an average age of 1-2 years are cut by a large knife about 0.6 meters above ground. These are shortened into 2-3 m long poles and then split into 1-1.5 cm thick sticks in the forest itself (Fig 2). These are rolled into bundles weighing about 2-3kg each and brought home. One person is able to make 2-3 bundles in a day.
- These bundles are soaked in water for one day. After soaking, each stick is splited again into thin sections. The required thickness is obtained by thinning them twice or thrice. These are then rolled in bundles and stored for 2-3 months until mat weaving is taken up. Generally it takes 2 days for one person to cut and make sufficient material for making one mat.
- Weaving of mat is done during summer season (July-September) and three different sizes of mats are made in these villages; big (2.3m x 1.8m), medium (1.8m x 1.4m) and small (1.4m x 1m) size. On average it takes about 2 days for a person to weave one mat of size 2.3m x 1.8m. However, sometimes the work may continue up to 10 days when it is done only during free time (say about 2 hours per day).
- The cane twines of 0.5 cm diameter are cut into long and thin strips and dried for about one month near the fire place for bordering the corners of the mats, thus giving the mat a final touch.

Fig 2. Mat making process

Seasonality of mat making

It has been observed that the mat-making business is seasonal. It is prevalent only during the rainy season when people cannot work in their field that is, during the months of June-September and during periods when there is shortage of work in the field *i.e.*, post harvest from the month of November to March. Preparation of raw material i.e., cutting, peeling and slicing of the bamboos is done from the month of November to March. This is also the season when bamboos mature and are ready for harvest. These thin slices of bamboos are then rolled in bundles and stored until the rainy season when mat weaving is taken up (Fig. 3).

Gender and age group involved in mat making business

The processing viz., splitting, slicing and weaving works for mat-making is tedious and mainly done manually by the female members of the family. On the other hand, cutting and transportation of bamboo is done by men folk. Young girls help elder women in processing of raw materials, while mat weaving is mostly done by older women of the age group 25 to 70 years. Seventy five percent of the women

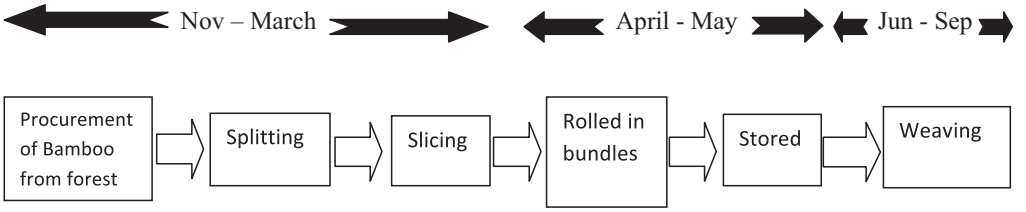

Fig 3. Step-wise process and seasonality of mat making in South Meghalaya

involved in mat making belong to the age group 25 to 45 years, 18% in 46 to 60 years and only 7% in the age group of 61 to 70 years.

Household involved and income from mat making

Mat making business is one of the important occupations in the studied villages. During the household survey, it was found that 70% of the households are engaged in mat making. Out of the four villages, Tangmang and Korblang villages have over 90% households involved in mat making, while in Nongskhen it is only 43% (Table 2). The average number of mats produced annually per household is 21. The value of standard deviation is large, reflecting a large spread in the number of mats produced annually. Village wise, the highest number of mats produced per household is at Korblang village and lowest at Nongskhen village (Table 3).

The input cost in mat making is low since bamboo as raw material is obtained for free from the forests or bought at a very low price i.e., Indian rupee INR 2/bamboo. The average annual requirement of bamboo per household for craft purpose was about 500 poles while that of cane was about 5 rolls of cane twine per household. A big size (2.3 m x 1.8 m) mat would require around 20-30 bamboos of 2 meter length and 5 cm diameter. The quantity of cane used per mat is also not so high. One roll of cane (18-20 meter long) costing INR 70/- per roll is sufficient for making borders of 8-10 mats. The cost of labour is not applicable since mat making is taken up during free time only or during the period when no employment is available. Transportation of the finished mats to the market is excluded from input cost as most of the mats are bought within the village itself by local traders. For one big size (2.3m x 1.8m) mat, the gross input is INR 67/-. Gross output is equal to the selling price from the producer which is INR 250/-, hence the net income for one mat is INR 183/-. Depending on the number of mat produced per household, the average net income per producing household per year comes to INR 3841/-. During the study period, it was found that mat making business contributes around INR 28 lakhs to the annual income of the villagers in the area (Table 3).

Markets

Mat making business has a ready market within the village and people can make fast cash out of it. Over 90% of the producing households sell their produce in their own homes, through the local traders or the middle men, while the rest sell them either in the local or in the town market mostly Pynursla and Shillong (Fig 4).

Table 2. Percentage of household engaged in bamboo mat making and quantity of mats produced annually.

Villages	HH involved in mat making business (%)	Mats produced /HH involved/yr (in Nos.)			
		Mean±SD	Minimum	Maximum	Sum
Tangmang	90	22.8± 15.2	4	60	616
Korblang	90	24.8±15.3	7	70	669
Mawdang	63	19.3±11.6	2	50	366
Nongskhen	43	11.9±11.7	3	40	154
All villages	**72**	**21.0±14.6**	**2**	**70**	**1805**

n = 120 households

Table 3. Annual income from bamboo mat making activity (in INR)

Villages	Mean±SD Income/producing households			Income/ household	Total income of village
	Gross output = No. of mats produced x Selling price	Gross input = Cost of bamboo + cane	Net income = (Gross output - Gross input)		
Tangmang	5704±3811	1529±1021	4175±2790	3758±2933	1352736
Korblang	6194±3897	1660±1044	4534±2852	4081±3035	326472
Mawdang	4816±2899	1291±777	3525±2122	2233±2404	444170
Nongskhen	2962±2917	794±782	2168±2135	939±1755	346769
All villages	**5654±3623**	**1406±976**	**3841±2666**	**2753±2845**	**2798613**

Bamboo Plantation and Management

In all four villages, bamboo grows both in wild and cultivated form. The important bamboo species mainly cultivated are *Bambusa jaintiana* and *Bambusa tulda*. The bamboo plantations are mostly monoculture and people follow a specific management system.

Planting of bamboo: For raising plantation the land is cleared by burning the forest during March-end and first week of April and bamboo is planted within few days after clearing. Burning operation is restricted only in those areas where plantation is to be raised. Two regeneration methods are usually practiced. The most common method is regeneration through rhizomes with culm-stock and roots. The young culms are kept intact, whereas for the old ones, the upper portion and branches are trimmed off. After cutting, these rhizomes are transported to the field and planted 20-30 cm deep in soil keeping a distance of 30 cm between the culms which are left uncovered by soil.

The other method is through culm cuttings where segments of one or two nodes from the lower portion of the culms are cut discarding the upper part and lateral branches. After cutting the segments are immediately transported to the plantation site and transplanted at 20-30 cm soil depth at a distance of 20-30 cm apart leaving the upper segment (about 10-15 cm) uncovered by soil.

The rhizomes and culms used for the plantation purposes are generally obtained from one year old bamboo. After one year the transplanted rhizomes start growing. However, growth through culm cuttings takes a longer period and has smaller diameter than that of those emerging from rhizomes during the first year of growth. Within 5-6 months after emergence, the bamboo attains its full height. The plantations are then allowed to grow and proliferate for 5 years.

Thinning: Thinning is done once in a year during rainy season (May – August). Both men and women are engaged in this activity. The branches of dense bamboo clumps are also

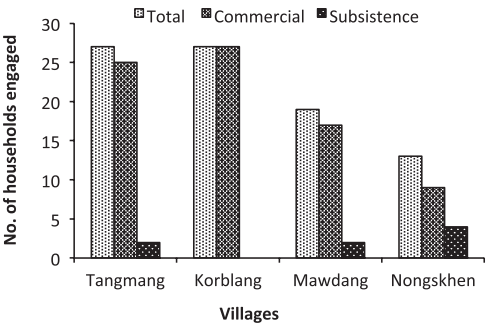

Fig 4. Households involved in commercial and subsistence mat making

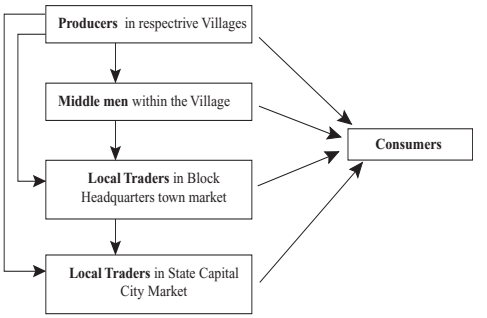

Fig 5. Households involved in commercial and subsistence mat making

trimmed and dead culms and branches are cut and removed to give space for new culms to grow.

Harvesting: Harvesting of bamboos is carried out during winter season i.e., from December – February. Men are usually involved in harvesting and transportation of bamboo poles. Bamboo poles are cut using a large knife (locally called as *'Wait'*) at about 0.6 m above the ground. For making mats, a straight well formed poles with an average age of 1–2 years are preferred. Transportation of bamboo from plantation site to collection site is usually carried out by collection teams comprising of 2–4 men.

Most of the peoples' needs for bamboo are met from their own plantations. Extraction from the plantation sites is strictly done at the time of needs. Bamboo groves ranging from 1-5 ha area are often rented to landless households for a period of 1-5 years from where they can extract bamboo for their household use. Young shoots of *Bambusa jaintiana* are not harvested for edible purposes since harvesting of young bamboo shoots would reduce the number of culms and in turn affect the availability of raw material for mat making.

DISCUSSION

The study revealed that bamboo mat making plays an important role in enhancing livelihood of rural poor as evident from the high percentage (72 %) of households engaged in this activity. Mat-making business is prevalent mainly during the rainy season and during post harvest periods when there is shortage of work in the field, thus, generating employment to sustain their family during times of crises. The number of mats produced per household varied significantly as evident from the large standard deviation. The annual income per household obtained from mat making activity was considerably lower than that obtained from sale of NTFPs like broomgrass (*Thysanolaena maxima*) or bayleaf (*Cinnamomum tamala*) or Packing leaf (*Phryrnium capitatum*) in South Meghalaya (Tynsong and Tiwari 2011, Tiwari 2005). Although, income from bamboo mat making was minimal in the area and cannot therefore constitute the sole source of income for majority of the households, the importance of such NTFP based activities particularly for poor households have been emphasized by many researchers (Falconer and Arnold 1989, Arnold 2002, Mbuvi and Boon 2009, Tynsong *et al.* 2012). Income from mat making is higher for villages with less livelihood options like Tangmang and Korblang, whilst people of Nongskhen and Mawdang are more engaged in trade and commerce. Women in the area share equal responsibility with men in looking after the family, although they earn much lower wages in comparison to men. For such section of society even a very small cash income matters a lot particularly if it is earned during a period when no other avenue of income is available. Similar studies by Shackleton and Campbell 2007, Tynsong and Tiwari 2011 found that sale of NTFPs assist weaker section of society in particular the women to cope with adversity.

Transportation facilities and update of market information is also a major constraint due to poor road connection. Therefore, most mat makers sell their finished products within their village itself through middlemen, who help the poor villagers overcome the vagaries of transport and uncertainties of market demand and supply. The growing commercialization of NTFPs over the past three decades has gained much attention in conservation circles as many a time it has led to unsustainable use and over exploitation of these forest resources (Arnold and Ruiz Pérez 2001, Ticktin 2004). However, in this case, the requirements of bamboo for mat making are met from bamboo plantations managed by the community. This plantation and management system has therefore sustained the supply of bamboo resources and also prevented species loss or environmental degradation from over-exploitation.

CONCLUSION

Bamboo mat making contributes significantly to the livelihood of people in the study area in terms of employment and cash income generation. Though only a subsidiary income, bamboo craft making have created employment opportunity particularly to the weaker sections of people during lean periods. In the case of

mat making, there is a gender balance in the share of work. The men are mostly involved in collection and transportation of bamboo culms from plantation sites to their homes, while the women are involved in processing activities and weaving of mats. Cultivation of bamboo in natural forest stands and proper management of these plantations have sustained the supply of bamboo resources for mat making since hundreds of years.

The study brings to fore the vast scope for enhancement of income of rural people by introducing better technology and marketing linkages. Keeping in view the potential of bamboo and the skills available with the people in bamboo craft making, there is a ample scope for development of bamboo based enterprises in the area. This will not only improve the economic status of the people already engaged in the business but it will also provide employment to many more people in the area.

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Cytokinin dynamics in cell suspension cultures of *Bambusa balcooa* Roxburgh using UPLC-ESI/MS/MS

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ABSTRACT

Tissue culture of plants is still much more art and skill than science. Rational Plant Tissue Culture involves the quantification of plant growth hormones in plants and media, to gain insight in kinetics and metabolism. We report on the use of UPLC-MS/MS for reliable and highly accurate determination of cytokinins in cell suspension cultures of *Bambusa balcooa*, growing on 2,4-D medium without cytokinins. The cytokinins are almost exclusively isoprenoid cytokinins, and the only aromatic cytokinin detected is BA, at very low concentrations. The predominant forms are glucosides and ribosides and the main cytokinins in cell suspension cultures are *trans*-zeatin-*O*-glucoside and *trans*-zeatinriboside-*O*-glucoside. Between days 8 and 14-16, when the growth of the cultures slows down the levels of *trans*-Z-*O*-G and *trans*-ZR-*O*-G decrease drastically, and from day 15 onwards up to 20 pmol of Z-N9-G is observed.

Keywords

Cytokinins, UPLC, Mass Spectrometry, cell suspension cultures, Bambusa balcooa

Abbreviations

2,4-D	2,4 dichlorophenoxyacetic acid
Trans-ZR	trans-zeatin-N9-riboside
Cis-ZR	cis-zeatin-N9-riboside
DHZR	dihydrozeatin-N9-riboside
DHZ	dihydrozeatin
DHZ-N9-G	dihydrozeatin N9-glucoside
Z-N7-G	zeatinN7-glucoside
Z-N9-G	Zeatin-N9-glucoside
iPG	N^6-isopentenyladenineglucoside
iPA	N^6-isopentenyladenosine
iP	N^6-isopentenyladenine
MS-iP	Methylthio-N^6-isopentenyladenine
MS-iPA	Methylthio-N^6-isopentenyladenosine
MS-Z	Methylthio-*trans*-zeatin
MS-ZR	Methylthio-*trans*-zeatinriboside
BA	benzyladenine
BAR	Benzyladenineriboside
BA(9)G	Benzyladenine-N9-glucoside
^{13}C-o-T	*ortho*-topolin
SPE	Solid Phase Extraction

INTRODUCTION

Bamboos and their distribution

Bamboo is one of the most useful plants to mankind. As a giant grass it has evolved to thrive under a variety of natural conditions, and consequently one finds bamboos all over the world from the northernmost part of Chili, up to the northern parts of China and Japan, and from sea level up to 3000-4000 meter (Himalaya, Andes). In most cases bamboos grow as part of forest ecosystems, but under certain conditions pure bamboo forests are well known.

It occurs worldwide with main centers of diversity in Asia and the Americas. In the New World over 400 different species have been described in South and Central America, from the North of Chili up to Mexico. In North America only one genus is endemic, namely *Arundinaria*, with three different species (Triplett *et al.*, 2006). In Africa the genetic diversity is relatively low (with large areas of *Arundinaria alpina* in Central Africa and of *Oxytenanthera abyssinica* in Eastern African countries like Ethiopia, Sudan), but from Madagascar over 40 different species have been described.

Bamboos come in many variants, from the tall woody bamboos in Asia, to climbing and clambering species, like *Dinochloa* (Wong, 1987) or various American bamboo species (Judiewicz *et al.*, 1999). Bamboos play a key role in forest dynamics because of their ability to take advantage of small or large-scale disturbances (Widmer, 1997).

Major movements of germplasm have occurred by human intervention, and as a consequence over 400 different types of bamboo, which are mainly ornamentals, occur in Europe and North America. The reintroduction of bamboo in Europe remedies its extermination during the last Ice Age. As another consequence, many Asian bamboos are found now throughout Africa and America.

Bambusa balcooa cell suspension cultures as model system

Bambusa balcooa Roxburgh is one of these examples. This species originates from Northeast India, and is originally found in Nepal, Bangladesh, Myanmar and Thailand. In South-Africa, it has become naturalized, where it is known as 'common bamboo'. It thrives under typical monsoonal climates with ample rain in the growing season (2500-3000 mm) and long dry seasons. It is one of the most useful species of bamboo supplying material for building and scaffolding, paper and pulp, boards and mats, handicrafts, and its young shoots are even consumed as vegetable.

As for most commercially interesting bamboos, hardly anything is known about their genetic basis (Gielis, 1999). Only recently, there has been some focus on exploring wild accessions of *Bambusa balcooa* in order to identify superior traits in terms of fiber quality among natural accessions (Bhattacharya *et al.*, 2010; Rai *et al.*, 2011).

Because of its economic potential *Bambusa balcooa* is also one of the species for which successful mass propagation methods through plant tissue culture have been developed, either via axillary branching (Gielis 1999; Das & Pal, 2005; Mudoi, K.D., Borthakur, M, 2009; Negi & Saxena, 2010), or via somatic embryogenesis (Gillis *et al.*, 2007). Cell suspension cultures have been derived from these embryogenic cultures.

Cell suspension cultures, in combination with feeding experiments, provide excellent model systems for studying biochemical pathways, developmental pathways (for example cell wall and microfibrils), gene expression and genetic transformation (Ojita *et al.*, 2011), or serve as a source of protoplasts. However, optimization of cell suspension cultures is essential if we wish to use these as model systems.

One of the key factors in this optimization strategy is the role of plant growth regulators PGR. Cytokinins and auxins are the key ingredients of culture media to control (1) the growth of cells and calli, (2) the regeneration and maturation of somatic embryos and (3) organogenesis, more specific branching and rooting, in micropropagation. However, little if anything is known about their uptake, metabolism and efficiency.

Understanding the metabolism of phyto-hormones may even be more critical for genetic transformation of bamboo. Despite many published or unpublished attempts, genetic transformation has hitherto been unsuccessful, other than mainly transient expression. Already in the early eighties it was

shown that *Agrobacterium* cells do not adhere to bamboo cells (Douglas *et al.*, 1985) and major deterrents to successful genetic transformation were only studied very recently (Sood *et al.*, 2011).

In this paper we present results on the optimization of cell suspension cultures as a model system and on the qualitative and quantitative analysis of the dynamics of endogenous cytokinins over time.

MATERIALS & METHODS

Bambusa balcooa cell suspension cultures and ploidy levels

The mother plant of *Bambusa balcooa* Roxb. originates from the Oprins Plant collection and has been kept at the Botanical Garden of Ghent. The same genotype has been used to induce somatic embryogenesis (Gillis *et al.*, 2007) and for the analysis of phytoactive components (Van Hoywhegen *et al.*, 2010). The cell cultures have been derived from somatic embryos originally induced from pseudospikelets of motherplants (Gillis *et al.*, 2007).

The cell cultures were cultivated on MS medium supplemented with 2,4-D 1,5 mgL^{-1} or 0,5 mgL^{-1} and 30 gL^{-1} sucrose. They grew in darkness at 25°C +/- 2°C on a liquid shaker (Innova 44 Incubator Shaker Series, New Brunswick Scientific).

For the callus cultures 2g/L^{-1} Gelrite (Duchefa) was added. For the experiments stocks of the cell culture were kept and each new experiment was initiated from these stock. Ploidy levels of cell suspension cultures were determined using methods described by Gielis *et al.* (1997).

Sample preparation for analysis

For the analysis 20 mL of cell culture solution was used, and the medium was separated from the cells by vacuum filtration (Whatman, filter paper Grade 12: 11 μm, cat. n° 1001-055.) After filtration the cells were ground in liquid nitrogen using a mortar and pestle.

Cytokinins (isoprenoid and aromatic), methylthio-cytokinins and cytokinin-*O*-glucosides were extracted from the samples using Bieleski mixture (Methanol/chloroform/formic acid/ water in 12/5/1/2 ratio) (Bieleski, 1964) which was added at 4mg/40 mg plant material at -20°C for 16 hours. After extraction, cell debris was removed by centrifugation (5min, 4°C, Eppendorf Centrifuge, 3220g). The resulting pellets were dissolved in 4 ml 80% Methanol HPLC grade and extracted again at -20°C for 45 minutes. After centrifugation the supernatant fractions were pooled and concentrated to waterphase with a rotavapor (Rotary Evaporator Büchi R110, Büchi water bath B-480 at 37°C). The water phase was diluted with 15 mL distilled water and adjusted to pH 7.

Solid Phase Extraction using DEAE-C18 and OASIS MCX columns

For the SPE with C18: Bond ElutR Solid Phase Extraction, Varian 6mL, 30/PK; 500 mg (Agilent, Santa Clara, CA, USA) was used. For the purification Solid Phase Extraction SPE was used with an anion exchange columns (DEAE Sephadex) and an inverted phase RP-C18 (LC-18 octadecyl bonded, Agilent) in series. The pH was adjusted to 7 and loaded onto the DEAE Sephadex-RP-C18 column. After rinsing with 20 mL doubly distilled water the RP-C18 column was uncoupled and eluted (Supelco Bulletin 910: Guide to Solid Phase Extraction) two times with 1,5 mL 80% methanol and 1 mL 100% methanol (20° HPLC grade methanol). Then the samples were dried at 27°C for 16 hours.

For the SPE with Oasis MCX columns (OASIS: Waters sample extraction products, OASIS MCX 6cc (150 mg) extraction cartridges (Waters, Milford, MA, USA) were used. During SPE all experiments were kept on ice during the procedure to prevent degradation of cytokinins.

When columns were almost dry, these were rinsed with 20 mL double distilled water. Then the column was rinsed two times with 2mL 100% methanol HPLC grade, followed by a rinse with 5mL of distilled water. The following step was the elution of the column with 1mL 5% NH_4OH in water for the elution of cytokinin phosphates and cytokinin-*O*-glucosides. A second elution step, with two times 1,5 mL 5% NH_4OH in 100% methanol HLPC grade was performed for the elution of isoprenoid and aromatic cytokinins and methylthio-cytokinins. To prevent adsorption of cytokinins silanylated glassware was used to capture the fractions. The fractions were dried using a Turbovap LV evaporator.

For both experiments the dried samples were dissolved in 100 µL HPLC-grade water and in 50 µL 100% HPLC grade methanol. Cell debris was removed by centrifugation (Microcentrifuge 5415D) for 3 minutes at 5,9g. The samples were dried under a continuous stream of nitrogen and then redissolved in 50 µL 10% methanol HPLC grade prior to the measurements. To compare both methods (DEAE-C18 versus OASIS) the experiments were performed according to standard protocols in triple. To be able to account for extraction efficiency and ionization efficiency internal standards were added (Olchemim, Olomouc, Czech Republic) (d-DHZR, d-DHZ, d-DHZ-N9-G, d-Z-N7-G, d-iPG, d-iPA, d-iP, d-MS-iP, d-MS- iPA, d-MS-Z, d-MS-ZR, d-BA, d-BAR, d-BA(9)G, ^{13}C-o-T, 20 pmol each).

UPLC-MS/MS Analysis

To measure the samples UPLC-MS/MS was used (Ultra High Pressure Liquid Chromatography, Acquity-TQD, Waters, Manchester UK). For data analysis Masslynx C V4.1 and Quanlynx (Waters, Manchester) were used. Separation is performed on a BEH-C18 column (Acquity UPLC BEH C18 1.7 µL, Waters Manchester UK) at 40 °C. 6 µliter of sample was injected at 4°C. The UPLC gradients are given in Table 1.

Mass spectrometry was executed on a TQC triple quadrupole mass spectrometer (Aquity-TQD, Waters, Manchester) with a positive electrospray ES^+ (cone 20-30V, source temperature 120°C, dissolvation temperature 450°C, cone gas flow 50l/hour, collision gas flow 0.20 L/min and collision energy 18-25 eV. The data were translated into the corresponding diagnostic transitions for Multiple Reactant Monitoring MRM.

For the statistical analysis a significance level of 5% was used. Because of the low number of replicates non-parametric tests were used.

Table 1

Time (min)	Flow Rate	% A (ammoniumacetaat)	% B (100% methanol)
Initial	0,3	100	0
7,5	0,3	58,3	41,7
9	0,3	33,4	66,6
9,1	0,3	0	100
10	0,3	0	100
10,1	0,3	100	0
12	0,3	100	0

RESULTS

Quantitative growth of cell suspension cultures

The fresh weight of the cell cultures is given in Figure 1. The growth and development follow a classic sigmoid curve. Starting from a fresh weight of 40 gL^{-1} the weight increases fourfold within ten days, and further increases to fivefold after 12 to 14 days.

Whereas in normal vegetative leaves the ploidy level was always 2n (Gielis *et al.*, 1997), in cell suspension cultures various ploidy levels are observed corresponding to 2n, 4n and 8n (Figure 1). No breakdown of DNA is observed. The composition of the population did not change significantly over time (results not shown).

Comparison of SPE methods

The comparison of the extraction efficiency of cytokinins between OASIS and DEAE columns for various cytokinins in cell cultures of *Bambusa balcooa* is displayed in Figure 2. The comparison was performed on both cells and the medium in which they grew. These measurements are the average of 5 replicates ± SD.

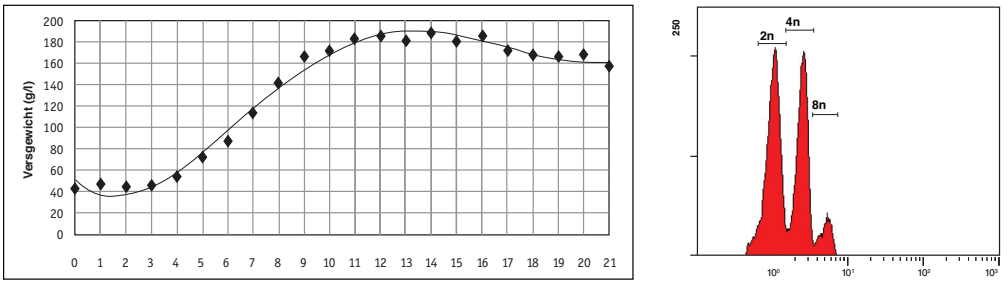

Figure 1: Left: Fresh weight of cell suspension cultures of *Bambusa balcooa* over a period of 21 days. Right: Flow cytometric analysis of the cell suspension culture.

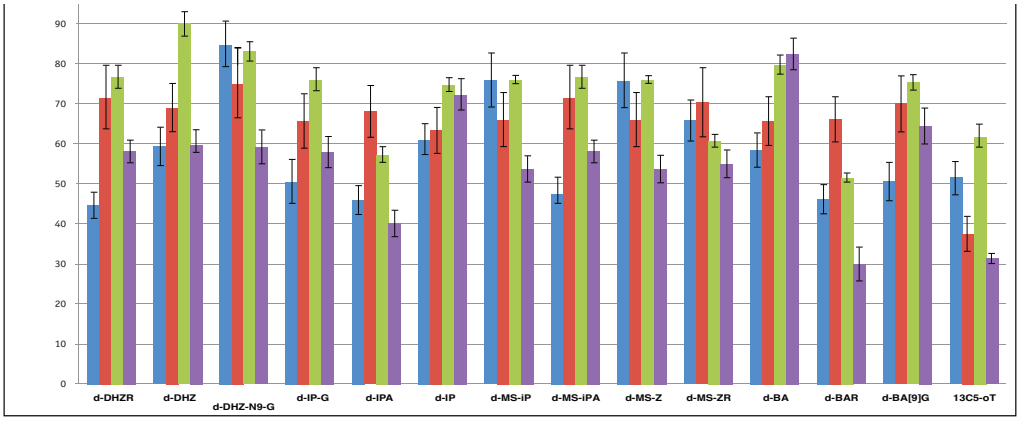

Figure 2: Extraction efficiency using OASIS and DEAE columns in cells and the medium.
■ = cells OASIS; ■ = cells DEAE; ■ = medium OASIS; ■ = medium DEAE

Cytokinin dynamics in cell suspension cultures

The predominant forms of cytokinins in cell cultures are *trans*-zeatin-*O*-glucoside and *trans*-zeatinriboside-*O*-glucoside (Figure 3). At day zero the *trans*-Z-*O*-G is very high, stabilizing around 20 pmol/g. Between days 8 and 14-16 the levels of *trans*-Z-*O*-G and *trans*-ZR-*O*-G decrease drastically. From day 9 to 12 a slight increase of *trans*-ZR is observed, but from day 15 onwards up to 20 pmol of Z-*N9*-G is observed. The predominant forms are glucosides and ribosides. Free bases are hardly present. The cytokinins are exclusively isoprenoid cytokinins. Aromatic cytokinins as BA are hardly above the detection limit.

DISCUSSION

A plant hormone or phytohormone is an organic substance other than a nutrient active in very minute amounts, which is formed in certain parts of the plant and which may be translocated to other sites, where it evokes specific biochemical, physiological and/or morphological responses. The classic groups of phytohormones comprise cytokinins, auxins, gibberellins, abscisic acid and ethylene (Davies, 2004). More recently, other phytohormones have been discovered including jasmonic acid, brassinosteroids, polyamines and strigolactones. All type of phytohormones are present and active in very small concentrations in plant tissue ranging between 0.01 – 1000 pmol/g fresh weight.

The qualitative and quantitative determination of cytokinins and cytokinin metabolism (and phytohormones in general) has been the focus of intense research efforts in the past two decades. HPLC is still one of the most widely used methods to separate cytokinins (Tarkowski, 2009), based on gradients in polarity (Chen, 1987). The identification and quantitative analysis of cytokinins is perfomed with Mass Spectrometry. The preferred method of analysis is the combination of LC with Electrospray Ionisation (Novak *et al.*, 2003). Tandem MS, ESI-MS/MS, is preferred for its increased sensitivity and lower background noise (Prinsen *et al.*, 2005).

In recent years Ultrahigh Performance Liquid Chromatography has come into focus or hormone profiling in plants (Kojima *et al.*, 2009). Compared to HPLC a UPLC column can withstand pressure up to 1000 bar, which ensures a very fast and improved separation of the components under study (Dolezal *et al.*, 2007; von Schwartzenberg *et al.*, 2007). Due to the superior separation, in combination with robustness, UPLC-MS/MS is considered as the most accurate and reliable method up to now. In this project we used UPLC coupled with mass spectrometry.

This method can be used to quantify all plant growth hormones, not only cytokinins, and can be developed into high-throughput

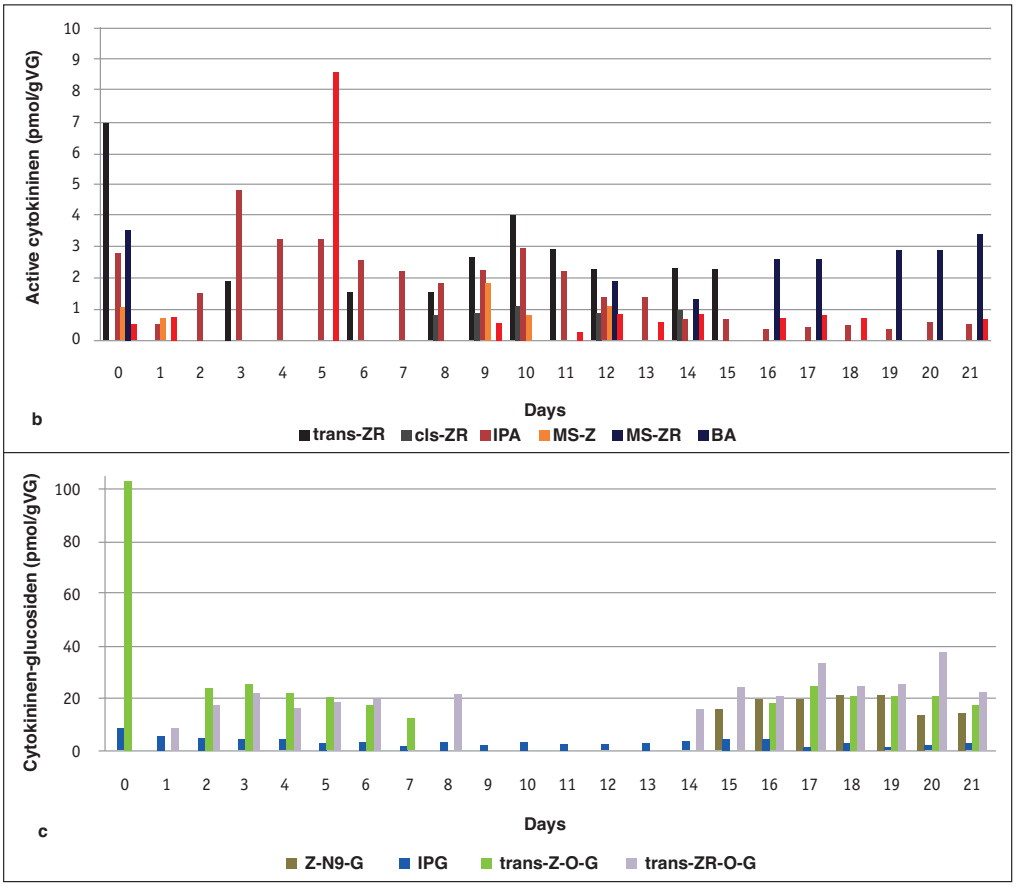

Figure 3: Kinetic profile of the endogenous cytokinins present in a *Bambusa balcooa* cell suspension culture during a 21 day culture period.

systems. We stress that the analysis is extremely sensitive and results, even within repetitions may greatly differ and differences are also found between two methods of SPE. Actually various parameters, apart from the different SPE methods, were extensively tested to optimize the method as a whole (results not shown), prior to the analysis of cell suspension cultures. Also prior to the analysis the cell cultures were grown for at least 3 subcultures under exactly the same conditions. The ploidy levels of the cells remained quite constant throughout the subculture with 2n, 4n and 8n peaks.

When the quantitative analysis was performed daily for the cell suspension cultures, a decrease of *trans*-Z-*O*-G and *trans*-ZR-*O*-G from around 20pmol/gFW to zero was observed when biomass increase in the cell suspension cultures slowed down. In the final week the original concentrations of these compounds were reached again, with a concomitant increase of Z-*N9*-G to about 20 pmol/gFW in the last week. IPG concentrations remained constant.

While this report is limited to cell suspension cultures, these methods were also used to quantify the cytokinin content in micropropagation, in leaves from mature bamboo and other species (monocots, poplar and willows) and to analyze cytokinin biosynthesis and metabolism in feeding experiments. These analytical methods allow for high-throughput measurements of cytokinins, auxins, gibberellins, abscisic acid and much more.

In mature bamboos the main cytokinins are isoprenoid cytokinins and mostly glucosides or ribosides. In *Fargesia rufa* (unpublished results) the glucosides IPG and Z-O-G were present at levels above 10 pmol/g FW (up to 60 pmol/gFW for Z-O-G). The only aromatic

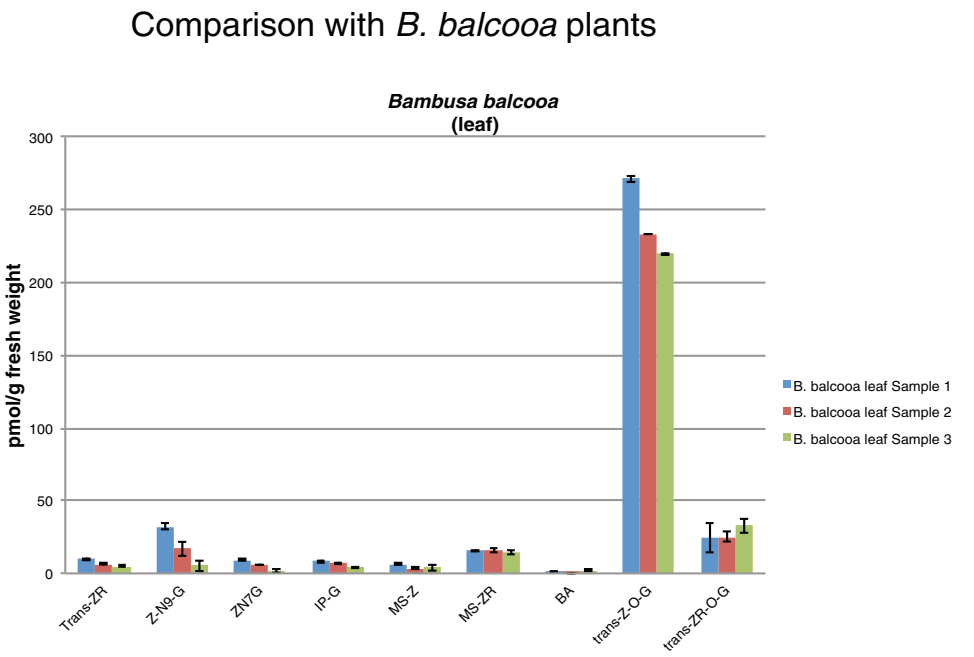

Figure 4: Levels of endogenous cygtokinins in three leaf samples of *Bambusa balcooa.*

cytokinins observed are the free base BA and its glucoside BA(7)G. In mature *Bambusa balcooa* (Figure 4) the main cytokinins found were *trans*-ZR, Z-N9-G (17 pmol/gFW), Z-*N7*-G, *trans*-Z-*O*-G (266 pmol/gFW) and *trans*-ZR-*O*-G (26 pmol/gFW) and the methylthio-forms MS-Z and MS-ZR (15pmol/gFW) and IPG. These measurements were based on 3 samples with 5 repetitions each.

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Bamboo – The plant and its uses. Book Review

Johan Gielis

Bamboo – The plant and its uses, edited by Walter Liese and Michael Köhl, is the 10th volume In the Springer Series *Tropical Forestry.* In 2015 Prof. W. Liese celebrates his ninetieth birthday, and almost 65 years of his life were devoted to bamboo research. Among many honorary doctorates and professorships, one of his most prestigious awards is the Brandis Medal. Sir Dietrich Brandis (1824-1907) is considered as the father of tropical forestry, but also wrote various papers on bamboo (Liese, 1986). In his last paper Brandis (1907) wrote: *"I believe I am justified to recommend the study of Bamboo to younger botanists. The main part of the work, however, must be done in countries where bamboos are indigenous, and the first operation must be to establish facts."*

Prof. Liese has brought this advice into practice, with many PhD students from Asia and a lasting influence in bamboo research in many countries in Asia, Africa and South-America. Foremost however has been his focus on establishing facts through research. We all know bamboo as a fantastic plant, which could provide solutions to many challenges mankind faces in the 21st century, from providing housing to a growing popu lation worldwide, all the way to mitigating climate change through reforestation and sustainable forestry. Without facts however, we are in the dark. This book aims to transfer knowledge and facts to younger generations.

Prof. Liese has bundled in this book much of his knowledge from 60 years of research on bamboo. His seminal work with his colleague Parameswaran on using electron microscopy to study the anatomy of bamboo culms and rhizomes, has led to a better understanding of preservation methods, increasing the lifetime of bamboo utilization, and of industrial processing methods. The chapters *Properties of the Bamboo Culm* and *Preservation and Drying of Bamboo,* written by him and his last PhD student Thi Kim Hong Tang, are highlights of the book. The final chapter on *Utilization of Bamboo* is quite complete, from more traditional uses in housing, the production of utensils and the use of the shoots, fruits and extractives, up to contemporary uses as bamboo parquet, bamboo-plastic composites, textiles and charcoal.

Prof. Ratan Lal Banik contributed three *chapters, namely Morphology and Growth, Bamboo Silviculture* and *Harvesting Techniques*. Also he bundles a lifelong expertise into those three very informative chapters. Although the main focus is on the Old World tropical bamboos, the detailed description of techniques and practices should be of interest to anybody involved in silviculture and agroforestry of bamboos. *Pest and Diseases of Bamboos* is another very informative contribution. The chapter on Priority Species however, is very short and this type of information can be found elsewhere.

The book focuses on stabilized scientific and technological knowledge and does not include ongoing research on physiology (see three chapters in this BSC volume) or DNA sequencing. Much of this research however is not stabilized yet and a lot of new results can be expected in the years and decades to come. One example is the first chapter of Lynn Clark, the leading expert on bamboo taxonomy today, describing the state of the art on Bamboo Taxonomy. The availability of molecular methods has led to many new insights in taxonomy but also raises many more questions. This is how science advances, and typically these advances are published in scientific journals. It is only when certain research areas have reached a certain maturity that it is time for a landmark book summarizing existing knowledge in a broad field. *Bamboo – the plant and its uses* is such a landmark book, and at the same time Prof. Liese's legacy, and is highly recommended to everyone involved in bamboo research.

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