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Original Article

Comparative Extraction of Chlorophylls in Selected Forest and Savanna Mosses Using Dimethylsulphoxide and Acetone

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Abstract

The present study compared the extraction of chlorophylls from selected forest mosses (*Hyophila involuta* and *Thuidium gratum*) and derived savanna moss (*Archidium ohioense*) using dimethylsulphoxide (DMSO) and 80% acetone. The mosses were collected from natural populations found in the Central Campus of the Obafemi Awolowo University, Ile-Ife, Nigeria. The chlorophyll extractions process followed standard methods and the absorbance of the extracts were read on spectrophotometer at wavelengths of 645 nm and 663 nm respectively. The data obtained were later subjected to appropriate statistical analysis. The results showed that DMSO was a better chlorophyll extractant for mosses than 80% acetone. Although there were significant differences in the chlorophyll *a*, chlorophyll *a/b* ratio and total chlorophyll accumulation within all three species using DMSO and 80% acetone as extractant ($P < 0.05$), there was no significant difference in the chlorophyll *b* accumulation of all the three species ($P > 0.05$).

Keywords: acetone, chlorophyll, dimethylsulphoxide, extractant, forest, savanna

Introduction

Bryophytes are the second largest group of land plants after flowering plants, comprising about 15,000 to 25,000 species worldwide (Gradstein *et al*., 2001). Members of this group have ancestral ties to the Chlorophyta (green algae), possessing chlorophyll *a* and *b* (Shaw, 2000). Chlorophyll, a natural pigment produced by green plants and algae, absorbs light energy from the sun, which is then used to synthesize carbohydrates from carbondioxide in a process called photosynthesis (Raven *et al*., 2005). Chlorophyll content can change in response to biotic and abiotic stresses such as pathogen infection (Mur *et al*., 2010) and light stress (Kitajima and Hogan, 2003; Brouwer *et al*., 2012).

Chlorophyll analysis has been conducted in numerous studies due to its importance in the physiology of plants. Thus, quantification of chlorophyll provides important information about the effects of environments on plant growth (Schlemmer *et al*., 2005). In addition to this, the extraction techniques of chlorophyll deserve special attention. It is necessary to have an accurate and efficient method to extract and measure chlorophyll from mosses. Even more, the location of chlorophyll in leafs makes the penetration of solvent and dissolution of chlorophyll a very complex process that is hard to predict and control (Shu-Mei *et al*., 2014). Currently, there are several methods for chlorophyll estimation. Some of the methods involve many procedural steps, which lead to dilution or loss of pigments. Extraction of chlorophyll in acetone has been the common method among bryologist (Wolf, 1958; Martin and Churchill, 1982; Glime and Keen, 1984; Penuelas, 1984). Raeymaekers and Longwith (1987) and Makinde and Akande (2012) used dimethylsulfoxide (DMSO) to extract chlorophyll from mosses and the method circumvented the difficulties arising due to the maceration using acetone. Most of the traditional methods of pigment analysis, including the often used High Performance Liquid Chromatography (HPLC) are not ideal to obtain long term data (Sims and Gamon, 2002).

The present work therefore intended to compare the extraction of chlorophyll contents from selected forest mosses (*Hyophila involuta* and *Thuidium gratum*) and derived savanna moss (*Archidium ohioense*) using dimethylsulfoxide (DMSO) and 80% acetone. The results from this work may also provide information about the changes that can be observed within mosses of the two vegetation zones.

Materials and Methods

Plant materials

The plant materials consisted in different moss species: *Archidium ohioense* Schimp ex. C. Muell, *Hyophila involuta* (Hook) Jaeg. and *Thuidium gratum* (Palis) Jaeg.; samples were collected from their natural populations, for a period of nine months, at the Obafemi Awolowo University (O.A.U) Ile-Ife campus, in Southwest of Nigeria (07°32'N; 04°31'E).

Chlorophyll extraction

The chlorophyll contents of the mosses shoots were extracted using 80% acetone according to the method of Raeymaekers and Longwith (1987) and absolute

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dimethylsulfoxide (DMSO) according to the method of Makinde and Akande (2012).

The extraction was done using 10 mg of each moss sample, placed in a test tube and 5 ml of DMSO added. Each test tube was covered with aluminum foil and thereafter incubated at 67 °C in the oven (Genlab Drying cabinet) for 15 hours. For acetone extraction, 10 mg of each moss sample were grounded in 80% acetone, with mortar and pestle after which about 0.8 g of sodium bicarbonate had been added. Following complete maceration, samples were filtered into test tubes using Whatman No.1 filter paper.

Spectrophotometric assays

The absorbance of the extracts was read on VIS S23A Spectrophotometer at wavelengths of 645 nm and 663 nm. The concentrations of chlorophyll *a* and *b* (mg/g) dry weight (DW) were determined using the formulae of Arnon (1949), respectively the total chlorophyll was calculated using the formula of Vernon (1960):

Chlorophyll $a = (12.7_{A663} - 2.7_{A645}) \times SW^{-1}$

Chlorophyll $b = (22.9_{A645} - 4.7_{A663}) \times SW^{-1}$

Total chlorophyll $= (6.45_{A663} + 17.72_{A645}) \times SW^{-1}$

Where: S = amount of solvent used for extraction (ml), W = weight of moss sample (mg).

Statistical analysis

Data obtained were analyzed using t-test to determine if there were significant differences between the two extratants studied at P = 0.05, while one-way analysis of variance (ANOVA) was used to determine whether there were significant differences between the three moss species.

Results

Chlorophyll extraction

The concentrations of chlorophyll *a* and *b* of *Archidium ohioense*, *Hyophila involuta* and *Thuidium gratum* from absolute DMSO and 80% acetone extracts are shown in Figs. 1, 2 and 3 respectively, while the concentrations of total chlorophyll and that of chlorophyll *a/b* ratios for the species under study are shown in Figs. 4, 5 and 6 respectively.

Chlorophyll *a*, *b*, *a/b* ratios and total chlorophyll concentrations in both DMSO and 80 % acetone extracts showed similar patterns, by recording lower values of chlorophyll concentrations between April and July for all the species studied. High values were later recorded, reaching the peak in October and thereafter declined in November, except that in *Thuidium gratum* chlorophyll *a/b* ratio that reached the peak in August and declined in September (Fig. 3). Also in *Hyophila involuta*, chlorophyll *a*, total chlorophyll and chlorophyll *a/b* ratio recorded peak values in September, and thereafter declined in October (Fig. 2).

Comparing the data for the three mosses species (Figs. 7 to 10) it was observed that *Thuidium gratum* had the highest chlorophyll *a*, *b*, total chlorophyll (6.89, 1.25, 8.32) mg/g dry weight (DW) and *a/b* ratio (6.56) respectively; it was followed by *Hyophila involuta* (5.01, 1.06, 7.60) mg/g DW, 4.71) while the least content was recorded by

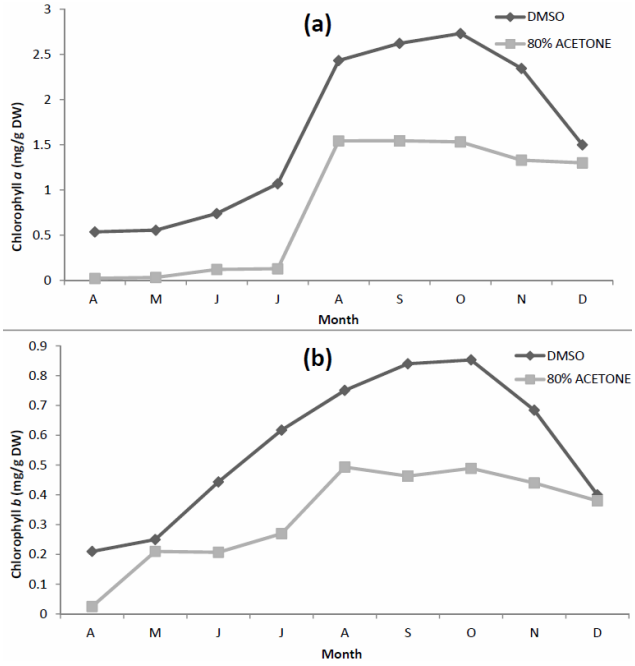

Fig. 1. (a) Time-course of changes in chlorophyll *a* concentration in *Archidium ohioense;* (b) Time-course of changes in chlorophyll *b* concentration in *A. ohioense*; A- April, M- May, ..., D- December

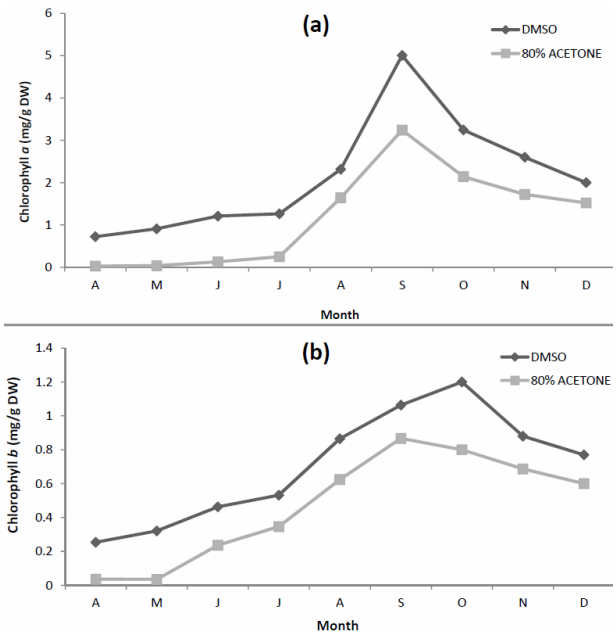

Fig. 2. (a) Time-course of changes in chlorophyll *a* concentration in *Hyophila involuta*; (b) Time-course of changes in chlorophyll *b* concentration in *H. involuta*; A- April, M- May, ..., D- December

Archidium ohioense (2.72, 0.85, 3.49) mg/g DW, 3.53). Low chlorophyll accumulation was recorded for all species between April and July, period that corresponded with the beginning of rainfall. High level of chlorophyll accumulation was recorded in the rest of the months and begun to decline between October until December, a period dominated by drought.

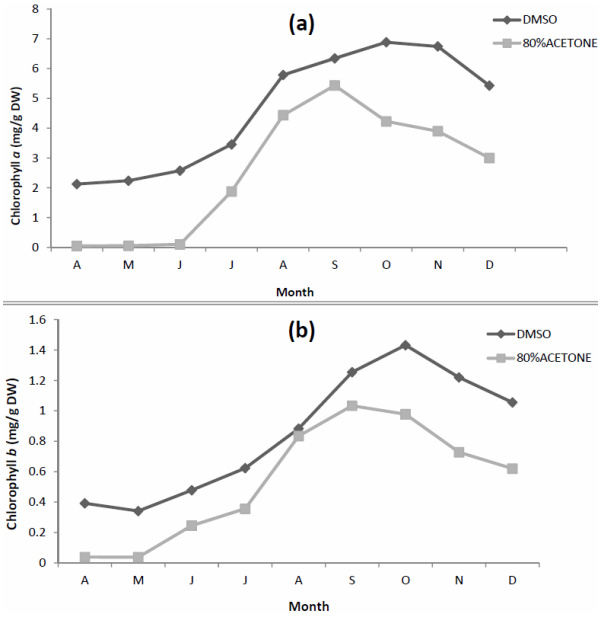

Fig. 3. (a) Time-course of changes in chlorophyll *a* concentration in *Thuidium gratum*; (b) Time-course of changes in chlorophyll *b* concentration in *T. gratum*; A- April, M- May, ..., D- December

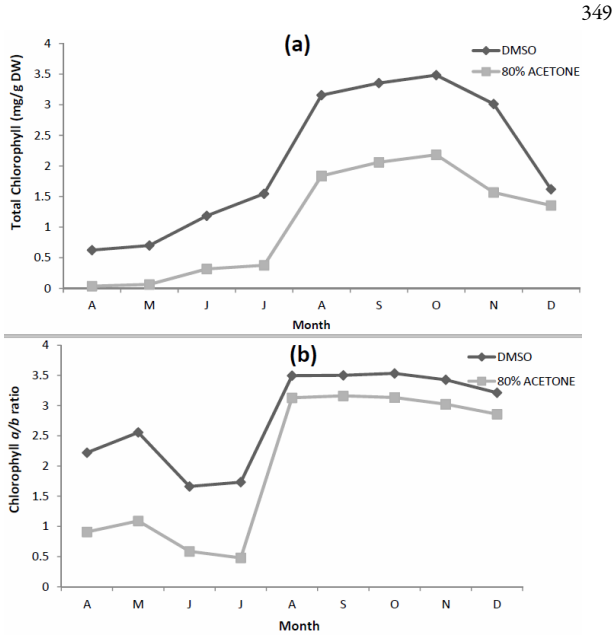

Fig. 4. (a) Time-course of total chlorophyll accumulation in *Archidium ohioense*; (b) Time-course of changes in chlorophyll *a/b* ratio in *A. ohioense*; A- April, M- May, ..., D- December

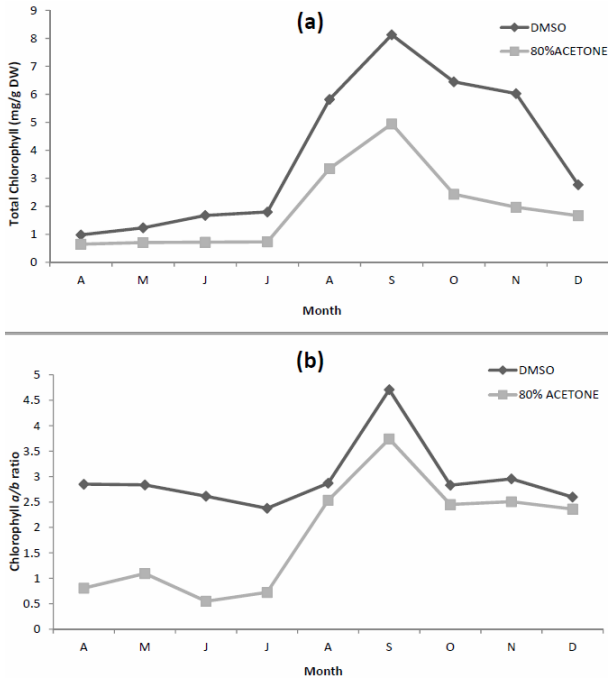

Fig. 5. (a) Time-course of total chlorophyll accumulation in *Hyophila involuta*; (b) Time-course of changes in chlorophyll *a/b* ratio in *H. involuta*; A- April, M- May, ..., D- December

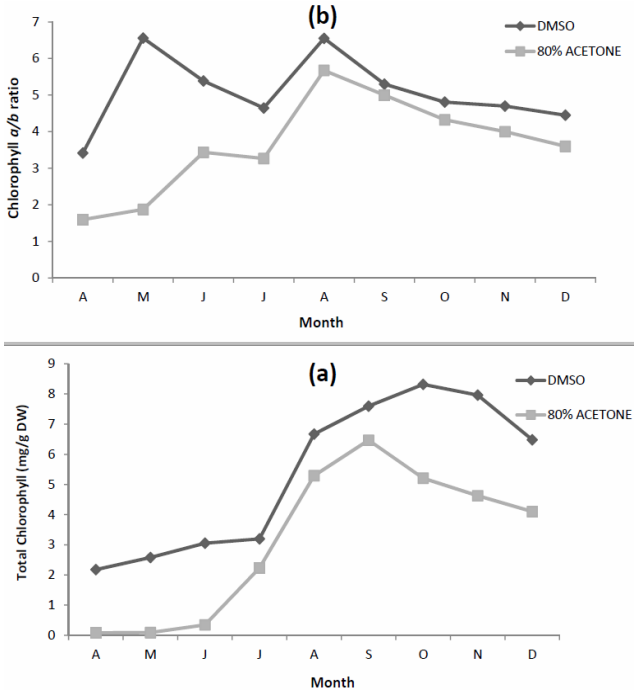

Fig. 6. (a) Time-course of total chlorophyll accumulation in *Thuidium gratum*; (b) Time-course of changes in chlorophyll *a/b* ratio in *T. gratum*; A- April, M- May, ..., D- December

Generally, DMSO chlorophyll extracts from savanna moss (*Archidium ohioense*) and the two forest mosses (*Thuidium gratum* and *Hyophila involuta*) recorded the highest chlorophyll *a*, chlorophyll *b*, chlorophyll *a/b* ratio and total chlorophyll content throughout the study period.

Statistical analysis

The results of the t-test showed that chlorophyll *a* and total chlorophyll accumulation observed within the two extractants were not significantly different ($P > 0.05$), while the chlorophyll *b* and chlorophyll *a/b* ratio noted were significantly different.

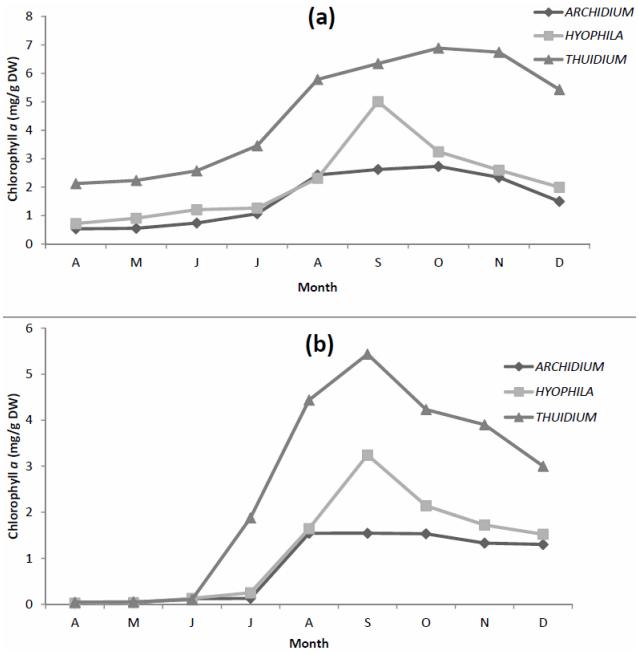

Fig. 7. (a) Time-course of changes in chlorophyll *a* concentration in dimethylsulphoxide extract for selected mosses species; (b) Time-course of changes in chlorophyll *a* concentration in 80% acetone extract for selected mosses species; A- April, M- May, ..., D- December

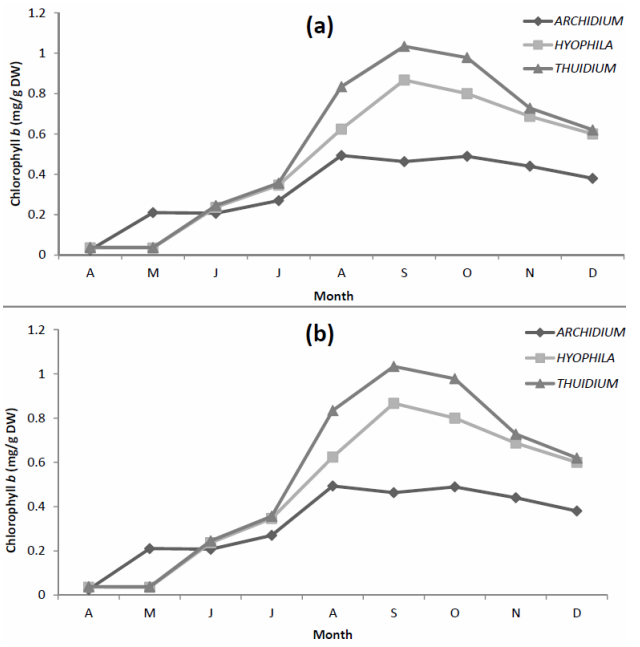

Fig. 8. (a) Time-course of changes in chlorophyll *b* concentration in dimethylsulphoxide extract for selected mosses species; (b) Time-course of changes in chlorophyll *b* concentration in 80% acetone extract for selected mosses species; A- April, M- May, ..., D- December

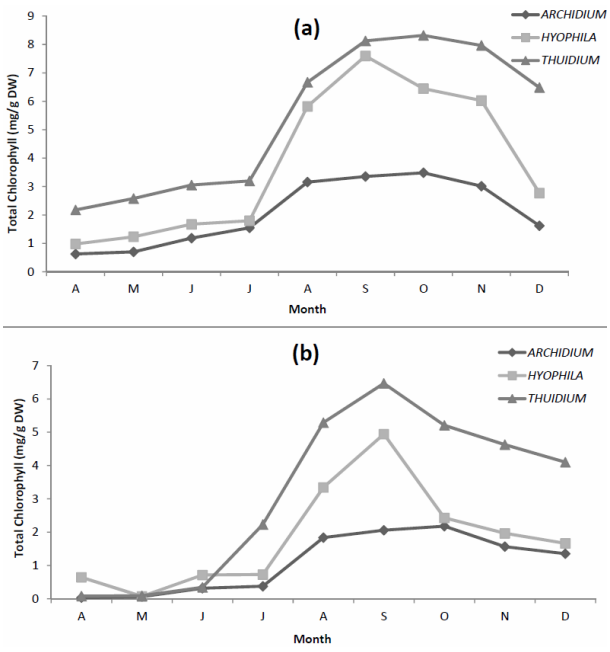

Fig. 9. (a) Time-course of total chlorophyll accumulation in dimethylsulphoxide extract for selected mosses species; (b) Time-course of total chlorophyll accumulation in 80% acetone extract for selected mosses species; A- April, M- May, ..., D- December

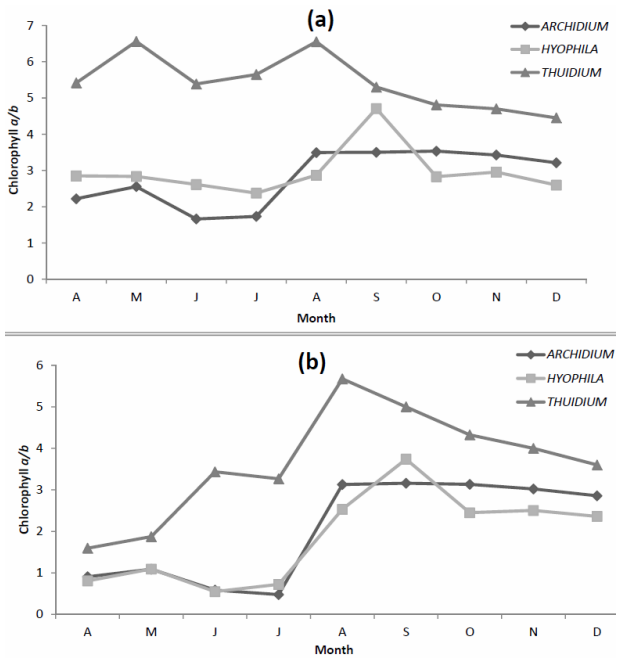

Fig. 10. (a) Time-course of changes in chlorophyll *a/b* ratio in dimethylsulphoxide extract for selected mosses species; (b) Time-course of changes in chlorophyll *a/b* ratio in 80% acetone extract for selected mosses species; A- April, M- May, ..., D- December

The result of the ANOVA showed that there were significant differences in the chlorophyll *a*, chlorophyll *a/b* ratio and total chlorophyll accumulation among all three species using DMSO and 80% acetone as extractant ($P < 0.05$) respectively. However, there was no significant difference in the chlorophyll *b* accumulation of all the three species ($P > 0.05$).

Discussion

The patterns of chlorophyll accumulation reported in this work clearly showed that DMSO is a reliable and reproducible extractant to extract chlorophyll in mosses. The method of extracting chlorophyll using DMSO does not require maceration of the plant samples, thus might increase the efficiency of the study. The elimination of this step reduced analytical errors and yielded a solvent with less turbidity, such as found in 80% acetone extract. DMSO gave a more complete extraction of chlorophyll pigments, which were also more stable over time than those with 80% acetone. This interpretation of the results agreed with those of Alberte *et al*. (1976), Raeymaekers and Longwith (1987). Bryophytes have relatively thick cells, relative to the size of protoplast, so the extraction of chlorophyll from them required a lot of time (Makinde and Akande, 2012). The submersion of these mosses in DMSO for 12 hours at 67 °C was found to be appropriate and efficient in this regard.

Water deficiency is known to cause reduced photosynthesis (Dilks and Proctor, 1974). Thus, a more intense chlorophyll accumulation was recorded within the current study during the rainy season and less chlorophyll occurred during the dry season. Di-Nola *et al*. (1983) also reported that chlorophyll contents of fresh sample of *Mniobryum* sp., *Barbula fallax* and *Tortula brevissima* were much higher than those in the dry states.

The accumulation of chlorophyll in *Thuidium gratum* and *Hyophila involuta* (forest moss species) was higher than that of the derived savanna mosses, *Archidium ohioense*. This could be due to the fact that these forest mosses possess leaves in which chlorocysts are sandwitched between several layers of large empty non-chlorophyllous hyalocysts, as a result of which the photosynthetic cells are protected from photo-oxidation (Fisher, 2006).

Boardman (1977) also reported higher chlorophyll concentrations in leaves of plants growing in environment characterized by low irradiances (e.g. in deep shade) relative to those in higher irradiances (e.g. open environment). Such changes in the characteristics of the chlorophyll content appear to represent adaptations to enhance the efficiency of light capture. Sluka (1983) also supported the concept of increased chlorophyll concentrations at low light intensities in bryophytes by showing that total chlorophyll content of mosses was inversely proportional with the light intensity. It has been widely accepted that photosynthetic pigments, mostly chlorophyll *a* and *b* tends to increase with decreasing irradiance in order to facilitate increased light harvesting in shade tolerant species (Givnish, 1988). In the present study, it was observed that the chlorophyll content increased in mosses under low light. High chlorophyll contents under low light situations found in *Thuidium gratum* and *Hyopila involuta* can therefore be related to their occurrence or distribution.

In terrestrial plants that grow within the shade of a leaf canopy, chlorophyll *b* concentrations increase more than those of chlorophyll *a* because chlorophyll *b* utilizes a slightly narrower wavelength span in accordance with the spectrum found there (Martin and Churchill, 1982; Marschall and Proctor, 2004). This also agreed with what was reported for seagrass (Sharon *et al*., 2011). The occurrence of lower chlorophyll *a/b* ratios in shade plants compared with sun plants was obtained as a consequence of chlorophyll *b* associated only with light harvesting chlorophyll protein (LHCP) complex of the photosynthetic unit, whereas chlorophyll *a* is found in LHCP and in other complexes and does not change in response to environmental conditions (Alberte *et al*., 1976). The low chlorophyll *a/b* ratio might also be as a result of large amount of light-harvesting chlorophyll protein (LHCP) complexes in the thylakoids of mosses (Aro, 1982b). LHCP complex have chlorophyll *a* and *b* in about equal amounts, whereas the photosystems (PS1 and PS11) have much more chlorophyll *a* than chlorophyll *b* (Salisbury and Ross, 1985). This explained the lower chlorophyll *a/b* ratio recorded by forest moss *Hyophila involuta* than the sun demanding species *Archidium ohioense*, in August, October, November and December.

Apart from the total chlorophylls, the ratio of chlorophyll *a* to *b* has been a key parameter to judge the shade tolerance of a particular species (Givnish, 1988); thus, shade tolerant species display a lower ratio under shade compared to their counterparts grown under high light environments. It has been shown that shade tolerant species produce a higher proportion of chlorophyll *b* relative to chlorophyll a, which leads to a lower chlorophyll *a/b* ratio, to enhance the efficiency of blue light absorption in low light environments (Yamazaki *et al*., 2005). Forest mosses in the present study responded in the opposite direction. Higher chlorophyll *a/b* ratio values were obtained under lower light intensities. This finding challenges the validity of using low chlorophyll *a/b* ratio as an indicator of shade tolerance of species in general.

Several studies reported decreased chlorophyll *a/b* ratio in response to shade (Kotzabasis *et al*., 1999), while a few studies reported an unchanged chlorophyll *a/b* ratios in the light gradient continuum (Murchie and Horton, 1998). Johnson *et al*. (1993) and Murchie and Horton (1998) showed only a weak association between chlorophyll *a/b* ratio and shade tolerance. Therefore, it is proposed that the changes in chlorophyll *a/b* ratio depending on the light environment might be a characteristic of species themselves. Increase in PS I units (size or number) explained the increased chlorophyll *a/b* ratio under low light, as PS I often does not contain chlorophyll *b* (Hirashima *et al*., 2006). On the other hand, increased number of PS II reaction centre also supports higher chlorophyll *a/b* ratio under low light.

The concept of sun and shade plants, which was developed with and for the leaves of vascular plants (Givnish, 1988; Larcher, 2003) does not seem to be fully coherent for mosses; they show corresponding patterns to some extent, but there is substantially more variation (Lovelock and Robinson, 2002; Marschall and Proctor, 2004). Water availability can be an important source of this

variability in mosses as exemplified by Ueno (2006).

Conclusions

Dimethylsulfoxide (DMSO) was a reliable and reproducible extractant to extract chlorophyll from mosses. The most chlorophyll accumulation was recorded during the rainy season. The accumulation of chlorophyll in *Thuidium gratum* and *Hyophila involuta* (forest moss species) was higher than that of the derived savanna mosses, *Archidium ohioense*. Higher chlorophyll accumulation in the forest moss species than that of the derived savanna mosses also provide information about the changes that may be observed in mosses of the two vegetation zones, with a possible strong influence from the water availability. Higher chlorophyll *a/b* ratio values were obtained in the current study under lower light intensities. These findings challenge the validity of using low chlorophyll *a/b* ratio as an indicator of shade tolerance of species in general, and enhance the need of further study mosses.

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