Chlorophyll *a* fluorescence measurements for validating the tolerant bryophytes for heavy metal (Pb) biomapping

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Chlorophyll *a* fluorescence measurement has been used as a probe to examine and compare the tolerance of bryophytes against heavy metals stress caused in field trials and under laboratory conditions. The ratio of variable fluorescence (F_v) to the maximal fluorescence (F_m) in dark-adapted leaves termed as 'maximal photochemical efficiency' of PS II (F_v/F_m) was measured in selected bryophytes to study their quantum efficiency. Tolerance potential for lead (Pb) in bryophytes was evaluated statistically by using Dunkun's Multiple Range Test, which indicates that lead-treated moss *Grimmia anodon* and liverwort *Riccardia pinguis* exhibited the most physiological damage of PS II. Conversely, minimal changes were observed in *Barbula vinealis* and *Thuidium cymbifolium.* Field trials of all the selected bryophytes exhibited moderate changes in the F_v/F_m ratio except *G. anodon* and *R. pinguis*. This variation in susceptibility is due to stress caused by metal pollution. Therefore, it is desirable to study the relative tolerance potential of bryophytes as little is known about their susceptibility to metals. Validated tolerant bryophytes species will have multiple applications in studies of biomapping, forest enrichment and carbon gain.

Keywords: Bryophytes, chlorophyll fluorescence, heavy metal, Pb tolerance.

HEAVY metals are widespread as environmental pollutants resulting from human activities. They are accumulated by some bryophytes without diminution of growth. An evaluation of bryophyte health is therefore required to assess their response to metal pollutants to validate tolerance. Bryophytes are widely used as biomonitors of atmospheric metal deposition[1–4] because of their ability to accumulate elevated concentrations of metals without impairing physiological parameters. Bryophytes are also known to suffer a decrease of chlorophyll content especially at low pH[5], thus affecting photosynthetic pigment concentrations and ultimately the vitality of the species depending upon their tolerance. However, this characteristic varies significantly from species to species. High concentrations of metals can also induce free radical toxicity[6] and cause damage of photosynthetic apparatus[7].

Photosynthetic efficiency of the bryophytes has been used to distinguish tolerant species. Several studies have reported that the decrease of F_v/F_m ratio is a reliable indicator of stress induced due to damage in plants[8]. Therefore, the use of chlorophyll *a* fluorescence (CF) for the detection of plant stress is a major area of research as the technique is rapid and measurements are non-destructive[9,10]. CF has been extensively used, both in fundamental studies of the photosynthetic mechanism *in vivo* and in the study of eco-physiological responses to the environment[11,12]. The intensity of CF from photosynthetic tissues provides a non-invasive signal that is used to determine the status of photosynthetic activity[13]. It estimates PS II activity, which is highly sensitive to conditions that impair photosynthesis because of environmental stress.

The present study aims at validating the tolerance potential to lead amongst several common bryophytes species (*Barbula vinealis*, *Thuidium cymbifolium*, *Plagiochasma appendiculatum*, *Polytricum piliferum*, *Dicranum undulatum*, *Bryum cellulare*, *Hylocomium splendeus*, *Grimmia anodon* and *Riccardia pinguis*) by measuring field and laboratory CF. To date, no attempt has been made to estimate changes in photosynthetic efficiency of bryophytes due to stress caused by this metal. It is the intention of this study to determine if the induced effects are identifiable using this plant stress detection method and if it is possible to isolate sensitive and tolerant species of bryophytes. The same information has applicability in biomapping the atmospheric quality by using validated tolerant species and to improve the efficacy of agricultural inputs as well as in the establishment of new forest cover.

Nine bryophytes were used. Moss patches were marked, identified and collected from the deep forest beds of Mukteshwar (lat. 29°28′N and long. 79°39′E) and were considered as a control for lead (Pb) treatment. Native analysis was done at different sites of Mukteshwar, i.e. deep forest cover, forest road side, urban sites (1 and 2 km) and in proximity to a bus stand. Study areas are situated at an altitude of 2265.1 m in Kumaon hill. The metadata during the experiments were measured for light (122 μmol m^{-2} s^{-1}), humidity (65%), soil pH (6.8) and temperature (12°C). Approximately the same size of thalli or moss colonies were selected in the field followed by sorting of healthy colonies after preliminary cleaning.

CF measurements were made in the field on bryophytes growing in their natural habitats at different sites. For this, bryophytes were dark adapted for 30 min by using the supplied leaf clips. To record, the sensor unit from the fluorimeter Plant Efficiency Analyser (PEA; Hansatech Instruments Ltd, King's Lynn, England) was held over the clip and the shutter blade opened to expose the leaf to a high irradiance beam of red LED light

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Table 1. Moss and liverwort species measured for chlorophyll *a* fluorescence (F_v/F_m) at different sites (rural and urban) of Mukteshwar for natural stress

Moss species	Forest cover	Forest road side	Urban site 2 km	Urban site 1 km	Bus stand
Barbula	0.803 ± 0.006[a]	0.800 ± 0.011[a]	0.738 ± 0.031	0.689 ± 0.010	0.594 ± 0.013
Thuidium	0.811 ± 0.021*	0.791 ± 0.006*	0.725 ± 0.021	0.667 ± 0.031	0.584 ± 0.007
Plagiochasma	0.779 ± 0.015[b]	0.769 ± 0.023[b,*]	0.734 ± 0.006*	0.670 ± 0.022	0.542 ± 0.027
Polytricum	0.831 ± 0.008	0.773 ± 0.021	0.705 ± 0.021	0.624 ± 0.020	0.572 ± 0.009
Dicranum	0.789 ± 0.005[c]	0.756 ± 0.004[c]	0.693 ± 0.011	0.632 ± 0.007[d]	0.547 ± 0.004[d]
Bryum	0.813 ± 0.032	0.795 ± 0.032	0.687 ± 0.004	0.644 ± 0.011	0.522 ± 0.031
Hylocomium	0.775 ± 0.007[e]	0.764 ± 0.003[e]	0.680 ± 0.007	0.653 ± 0.030	0.537 ± 0.005
Grimmia	0.790 ± 0.021	0.698 ± 0.013	0.575 ± 0.020	0.300 ± 0.010	0.061 ± 0.008
Riccardia	0.762 ± 0.009	0.531 ± 0.006	0.401 ± 0.011	0.204 ± 0.004	0.099 ± 0.017

Values are represented as mean ± SE.
Significance test of ANOVA and DMRT has been done at 1% and 5% significance level.
Values superscripted with the same alphabets in horizontal row are not significantly different at 1% and 5% significance level at different sites.
*Values in horizontal row are significantly different at 5% significance level.

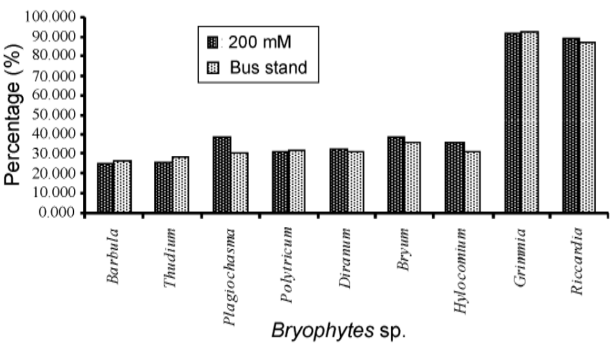

Figure 1. Comparative graph of percentage decrease in F_v/F_m ratio of different bryophytes at maximum concentration (200 mM) over control and bus stand over Mukteshwar forest cover.

(maximum 3000 μmol m^{-2} s^{-1}) during the measurement. At the completion of measurement (1 s) the calculated parameters of F_Φ, F_v, F_m, F_v/F_m and area above the curve were automatically displayed and stored to instrument memory.

Similar sized specimens of bryophytes collected from natural habitats were brought to the laboratory within 24 h and kept in petri dishes. Control and treatments of lead acetate (5, 50, 100 and 200 mM) were applied in a closed chamber (45 × 66 cm) and CF was measured after a 30 min dark adaptation.

Samples were analysed in triplicates to conduct statistical analysis. Values were represented as mean ± standard error[14]. ANOVA revealed significant differences in the F_v/F_m values of the bryophytes in different concentrations (for $P \leq 0.01$, $P \leq 0.05$) utilizing Dunkun's Multiple Range Test[15]. The results are presented on the basis of statistical variability in bryophytes tolerance potential with respect to heavy metal concentrations.

Measurement of F_v/F_m ratio data remained nearly constant in all the selected bryophytes growing under forest cover (Table 1) in native conditions. Many of these figures fall within a close range, i.e. 0.831–0.700 for bryophyte species under forest cover and road side areas. By comparison, marginally lower values were measured for bryophytes growing in urban sites (1 and 2 km) and in proximity to the bus stand. However, F_v/F_m values of bryophytes growing under forest cover and close to the bus stand exhibited significantly different ($P \leq 0.01$, 0.05) results in all the studied mosses and liverworts, which confirms the effects of response to different kinds of environmental stresses on photosynthetic functions. Data as a whole did not exhibit lower F_v/F_m values in most of the bryophytes except *G. anodon* and *R. pinguis* which exhibited minimum values as 0.061 and 0.099 respectively, growing near the bus stand (Figure 1).

An attempt was made to understand the effect of Pb on the photosynthetic apparatus status of bryophytes. It was observed that with the increase in concentration of heavy metal (Pb), the F_v/F_m ratio decreased linearly (Table 2). CF remained nearly constant in most of the moss species treated by 5 mM of Pb solution. The decrease in the ratio was maximal in *G. anodon* (91.8%) and *R. pinguis* (89.3%), whereas the minimum was in *Barbula vinealis* (25.1%) and *T. cymbifolium* (25.4%) in 200 mM concentrations over control (Figure 1). Non-significant ($P \leq 0.01$, 0.05) results were observed in some bryophytes up to 50 mM lead concentration, whereas the same was significantly different and more pronounced at 5% at 100 mM in *T. cymbifolium*. The inhibition rate of photosynthesis was measured by feeding different concentrations of Pb to *G. anodon* and *R. pinguis*.

In forest cover high F_v/F_m ratios reflect that under undisturbed and healthy habitats, plants undergo normal physiological functions in both tolerant and sensitive moss species. The F_v/F_m ratio in natural habitats ranged between 0.831 and 0.700 in forest cover; there were substantial differences in F_Φ over the area of hydrated bryophytes that could be considered as striking species characteristics[16]. The same was found minimum in moss

Table 2. Moss and liverwort species validated for Pb tolerance by measuring chlorophyll *a* fluorescence (F_v/F_m) in laboratory after 30 min treatment for metal stress

Moss species	Control	5 mM	50 mM	100 mM	200 mM
Barbula	0.812 ± 0.040^{a}	0.810 ± 0.011^{a}	0.764 ± 0.020	0.679 ± 0.005	0.608 ± 0.016
Thuidium	$0.803 \pm 0.004^{b,c}$	$0.799 \pm 0.021^{b,d,*}$	$0.775 \pm 0.007^{c,d}$	0.711 ± 0.011*	0.599 ± 0.022
Plagiochasma	0.809 ± 0.007	0.781 ± 0.043	0.746 ± 0.011	0.630 ± 0.005	0.502 ± 0.004
Polytricum	0.710 ± 0.016	0.640 ± 0.024	0.605 ± 0.005	0.531 ± 0.006	0.490 ± 0.020
Dicranum	0.749 ± 0.010	0.713 ± 0.006	0.610 ± 0.014^{e}	0.609 ± 0.021^{e}	0.510 ± 0.014
Bryum	0.815 ± 0.012*	0.808 ± 0.014*	0.602 ± 0.004	0.540 ± 0.006	0.501 ± 0.007
Hylocomium	0.794 ± 0.003	0.771 ± 0.008	0.705 ± 0.021	0.601 ± 0.018	0.509 ± 0.002
Grimmia	0.750 ± 0.040	0.610 ± 0.012	0.401 ± 0.016	0.204 ± 0.020	0.061 ± 0.011
Riccardia	0.741 ± 0.032	0.563 ± 0.008	0.479 ± 0.011	0.031 ± 0.014	0.079 ± 0.017

Values are represented as mean ± SE.
Significance test of ANOVA and DMRT has been done at 1% and 5% significance level.
Values superscripted with the same alphabets in horizontal row are not significantly different at 1% and 5% significance level at different sites.
*Values in horizontal row are significantly different at 5% significance level.

collected near the bus stand. It confirms that environmental stress parameters had an adverse effect and the same was suggested by the increase in fluorescence. The main sources of stress at urban sites and near the bus stand were derived from increasing vehicular traffic, tourist activity, leaded batteries, wear and tear of tyres and motor parts[17]. Pollutant discharge from local sources in densely areas of city also plays a major role in the increase of metals resulting in pollutant load[18] that could diminish the absorption characteristics and consequently, CF. Stress by pollutants including metal precipitation could be the cause of inhibition of PS II efficiency in some species which can be identified as sensitive species. Therefore, the present findings are in agreement with[19] the described adverse effects of metals on CF parameters. Since the physiological state of the photosynthetic apparatus differed at different tested sites (forest cover, forest road side, urban sites and bus stands), we believe that, the heterogeneity of CF may reflect the quality of the growth zone.

It was evident that the bryophyte behaviour at different locations may be due to atmospheric influence. However, values which range from 0.8 to 0.7 show nearly identical behaviour. and indicate a similar physiological state of the photosynthetic apparatus and a favourable habitat. By contrast, the F_v/F_m value of *G. anodon* and *R. pinguis* quickly changed upon treatment and was lower even upon a slight change of location.

Using F_v/F_m values as a screening parameter, it was possible to distinguish stress induced by Pb within 30 min thus, to identify the tolerant species or status of photosynthetic efficiency. Pb has a strong effect on chloroplasts but most of the Pb is sequestered as phosphate precipitation[20,21] or bound to the cell wall[22] and is responsible for membrane damage and leakage in bryophytes[23]. F_v/F_m ratio remained nearly constant in most of the moss species at lower concentration (5 mM), revealing that the maximal photochemical capacity of the photosynthetic cells remains unchanged at lower concentration.

PS II activity (presented by F_v/F_m) was observed to decrease due to Pb toxicity, which indicates that this metal strongly intervenes in photosynthetic electron transport. When metal concentration was increased to 50 mM, the F_v/F_m values start to decrease, whereas the decrement was more pronounced at 100 and 200 mM concentration. Present results affirmed that *G. anodon* and *R. pinguis* seem to be the least tolerant to metal stress of the studied bryophytes as evidenced by the high fluorescence signal, i.e. lower F_v/F_m values (Table 2). However, *Barbula vinealis*, *T. cymbifolium* and *Bryum cellulare* are comparatively more tolerant bryophyte species and are recommended for biomapping as well as use in forest enrichment.

The present results suggest multiple applications of CF which can be applied as a useful tool[24] to follow the response of bryophytes to environmental factors and also in validating tolerant species.

1. Rühling, A., Atmospheric heavy metal deposition in Europe – estimations based on moss analyses. *NORD*, 1994, **9**, 1–53.
2. Markert, B., Herpin, U., Berlekamp, J., Oehlmann, J., Grodzinska, K. and Mankovska, B., A comparison of heavy metal deposition in selected Eastern European countries using the moss monitoring method, with special emphasis on the Black Triangle. *Sci. Tot. Environ.*, 1996, **193**, 85–100.
3. Fernandez, J. A. and Carballeira, A., Biomonitoring of metal deposition in Galicia (NW Spain) with mosses: factors affecting bioconcentration. *Chemosphere*, 2002, **46**, 535–542.
4. Saxena, D. K., Singh, S. and Srivastava, K., Metal precipitation in Garhwal hill area (India): Estimation based on native moss analysis. *Aerosol. Air Qual. Res.*, 2008, **8**, 94–111.
5. Garty, J., Karary, Y. and Harel, J., Effect of low pH, heavy metals and anions on chlorophyll degradation in the lichen *Ramalina duriaei* (De Not) Bagl. *Environ. Exp. Bot.*, 1992, **32**, 229–234.
6. Gadd, G. M., Interaction of fungi with toxic metals. Tansley Review no. 47. *New. Physiol.*, 1993, **124**, 25–60.
7. Kalaji, M. H. and Loboda, T., Photosystem II of barley seedlings under cadmium and lead stress. *Plant Soil Environ.*, 2007, **53**, 511–516.
8. Cartor, G. A. and Miller, R. L., Early detection of plant stress by digital imaging with in narrow stress sensitive wavebands. *Remote Sensing Environ.*, 1994, **50**, 295–302.

9. Jones, H. G., *Plants and Microclimate*, Cambridge University Press, 1992, 2nd edn.
10. Bolhar-Nordenkampf, H. R. and Oquist, G., Chlorophyll fluorescence as a tool in photosynthesis research. In *Photo-synthesis and Production in a Changing Environment* (eds Hall, D. O. *et al.*), Chapman and Hall, London, 1993, pp. 193–206.
11. Lichtenthaler, H. K. (ed.), *In vivo* chlorophyll fluorescence as a tool for stress detection in plants. In *Applications of Chlorophyll Fluorescence*, Kluwer, Dordrecht, 1988, pp. 129–142.
12. Gries, C., Lichens as indicators of air pollution. In *Lichen Biology* (ed. Nash, T. H.), Cambridge University Press, Cambridge, 1996, pp. 240–254.
13. Govindjee, Sixty three years since Kantsky: Chlorophyll *a* fluorescence. *Aust. J. Plant Physiol.*, 1995, **22**, 131–160.
14. Snedecor, G. W. and Cochran, W. G., *Statistical Methods*, Iowa State University, USA; Oxford and IBH Publishing Co, New Delhi, 1967.
15. Karmer, C. Y., Extension of multiple range tests to group means with unequal numbers of replication. *Biometrics*, 1956, **12**, 307–310.
16. Takacs, Z., Otvos, E., Lichtenthaler, H. K. and Tuba, Z., Chlorophyll fluorescence and CO_2 exchange of the heavy metal treated moss, *Tortula ruralis* under elevated CO_2 concentration. *Physiol. Mol. Biol. Plants*, 2004, **10**, 291–296.
17. Asthana, D. K. and Asthana, M., *Environment: Problems and Solutions*, S. Chand and Company Ltd, New Delhi, 2005, pp. 166–181.
18. Saxena, D. K., Singh, S. and Srivastava, K., Biomapping studies from India: use of moss *Isopterygium elegans* for measuring seasonal metal precipitation in Garhwal by transplant technique. In *Bryology in the New Millennium* (eds Haji Mohamed *et al.*), Malaysia, 2008, pp. 343–356.
19. Singh, M., Turn-over of D1 protein encoded by *psbA* gene in higher plants and cynobacteria sustains photosynthetic efficiency to maintain plant productivity under photo inhibitory and non-photo inhibitory irradiance. *Photosynthetica*, 2000, **38**, 161–169.
20. Koeppe, D. E., Lead: Understanding the minimal toxicity of lead in plants. In *Effect of Heavy Metal Pollution on Plants* (ed. Lepp, N. W.), Applied Science Publishers, London, 1981, chap. 2.
21. Lichtenthaler, H. K. and Rinderle, U., Role of chlorophyll fluorescence in the detection of stress conditions in plants. *CRC Crit. Rev. Anal. Chem.*, 1988, **1** (19 Suppl.), 29–85.
22. Otero, S., Núñez-Olivera, E., Martínez-Abaigar, J., Tomás, R., Arróniz-Crespo, M. and Beaucourt, N., Effects of cadmium and enhanced UV radiation on the physiology and the concentration of UV-absorbing compounds of the aquatic liverwort *Jungermannia exsertifolia* subsp. *Cordifolia*. *Photochem. Photobiol. Sci.*, 2005, **5**, 760–769.
23. Brown, D. H. and Buck, G. W., The cellular location of metals in two bryophytes and lichen. *Cryptogamie, Bryol. Lichenol.*, 1985, **6**, 279–286.
24. Raua, S., Miersch, J., Neumann, D., Weber, E. and Krauss, G.-J., Biochemical responses of the aquatic moss *Fontinalis antipyretica* to Cd, Cu, Pb and Zn determined by chlorophyll fluorescence and protein levels. *Environ. Exp. Bot.*, 2007, **59**, 299–306.

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Unique graft combination of tea, Cr-6017/UPASI-9

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A unique graft combination in tea comprising a quality clone Cr-6017 as a scion and drought hardy high-yielding clone UPASI-9 as root stock was studied under commercial conditions. Average survival rate of cuttings of grafted plants in the nursery was above 85%. Grafted plants of Cr-6017/UPASI-9 were more vigorous and irrespective of the year from planting registered significantly high yields over ungrafted controls. Grafting resulted in increase in the number of branches, branch length and reduction in the branch angle. Grafted plants registered significantly low values of mesomorphy index, vulnerability index, shoot water potential and high values of F_v/F_m. Grafted and ungrafted Cr-6017 plants registered significantly high quantities of total polyphenols and catechins. Variation in the biochemical composition of green leaf and made tea of grafted and control plants of Cr-6017 was statistically non-significant. Quality attributes of teas manufactured from Cr-6017 were superior compared to teas from UPASI-9.

Keywords: Anatomy, biochemical composition, bush architecture, Cr-6017/UPASI-9, drought tolerance, nursery grafting.

GRAFTING has been practised for a long time all around the world. Plantation crops like rubber and horticultural crops like apple, pear, plum, and other woody perennials received most attention in this aspect[1]. In the case of tea, until about 50 years ago, seeds were the only source of propagation. In the absence of suitable mode of vegetative propagation, grafting was tried in the beginning of the last century by certain tea-growing countries to propagate the selected clones primarily for the production of seeds[1–4]. In the early thirties, a simple and economic method of vegetative propagation, in which capacity to root and rapidly form satisfactory nursery plants from single nodal cuttings from the young shoots was demonstrated[5]. Although it took some time to standardize the practice, the method has been widely used since 1950s by most of the tea-growing countries of the world.

Earlier work on grafting in tea mainly aimed at induction of more number of flowers in a shorter time for the production of seeds[1,3,4]. In the case of budding and grafting methods followed in tea, the rootstock had been either established in the field or a seedling from the nursery. During 1971, a unique method was demonstrated for cleft

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