B2_018_PF: THE INFLUENCE OF WATERING SOIL AND THALLUS ON PHYSIOLOGICAL PROCESSES AND NATURAL PRODUCTS OF THE TRANSPLANTED LICHEN *Parmotrema tinctorum*

Mongkol Phaengphech,[1]* Prichukorn Khongsatra,[2] Wetchasart Polyiam,[1] Chutima Sriviboon,[2] Kansri Boonpargob[1]
[1]Department of Biology, Faculty of Science, Ramkhamhaeng University, Bangkok, Thailand
[2]Department of Chemistry, Faculty of Science, Ramkhamhaeng University, Bangkok, Thailand
*e-mail: mongkolpp@gmail.com

Abstract: Lichen produces secondary metabolites that are different from other organisms and have great potential to be utilized commercially. However, lichens have extremely low growth rate due to their photosynthesis active only during high atmospheric humidity, which only exist in early morning. The objective of this study was to transplant the lichen *Parmotrema tinctorum* on man-made substrate, and provide extra water through soil watering, and direct spraying on thallus. It was hypothesized that additional water could enhance lichen production. The transplantation was conducted at Khao Yai National Park by using 200 fragmented thalli. They composed of four treatments: 1) without soil watering-without thallus watering (SD-TD), 2) without soil watering–thallus watering (SD-TW), 3) soil watering–without thallus watering (SW-TD), 4) soil watering–thallus watering (SW-TW). After a year of transplantation, we found that the lichens received extra water through soil watering and thallus spraying (SW-TW) had the highest growth rate measured 9.85 mm y^{-1}, while the highest biomass production of 56.4 μg g^{-1} was measured from soil watering alone (SW-TD). Contrary, photosynthesis (P_N) and Fv/Fm of both treatments declined. More importantly, daily soil watering alone (SW-TD) without thallus spraying, increased growth, biomass and P_N of the lichens more than only thallus spraying (SD-TW), once in two weeks. This treatment should be taken into consideration for future transplantation as well.

Lecanoric acid was the main natural product that *P. tinctorum* metabolized. This substance and methyl orsellinate, the minor substance, declined in all treatments after transplantation. Contradictory, orsellinic acid increased in all treatments. Whereas, atranorin, the second main substance, had varying amounts. It is most essential to continue research and development on lichen transplantation to achieve the maximum production of lichens by using the information from this study as a basis.

Introduction: Lichens produce novel products that are different from other organisms. They are used in pharmaceutical industries, cosmetic, foods, beverages and etc.[1] They are widely used as bio-indicator of air pollution. However, lichens grow extremely slow, which is not sufficient to utilize sustainably. Lichens depend on atmospheric moisture for carbon assimilation. Their photosynthesis terminate in early morning when relative humidity drop.[2,3,4] Our research question was, whether extra water supplied to thallus through soil watering, and spraying directly over thallus can enhance lichen production. We hypothesized that 1) additional supply of water could increase growth rate, biomass production, photosynthesis and vitality of lichens because water is one of the raw materials for organic matter production 2) spraying water directly over thallus increases growth and biomass more than water vapor from soil because thallus absorbs liquid water better than water vapor or atmospheric moisture.

The lichen *Parmotrema tinctorum* was used as the experimental material. This lichen distributes in all ecosystems in Thailand, and found abundantly at Khao Yai National Park.[2] It has been used successfully for silk dying and bio-indicator of atmospheric quality in the country. It also metabolizes secondary substances that have potential to be used pharmaceutically and much more.[1] Our objectives were to improve lichen production by transplantion the lichen *P. tinctorum* on man-made substrate, and supply additional water through water evaporate from soil watering, and spraying water directly over thalli.

Methodology:

Transplantation site: The secondary forest, previously a tropical rain forest, at Khao Yai National Park (KYPN), Thailand was used as the experiment ground.

Transplanted method and sample collection; The transplanted thalli included a few peripheral lobes of about 3-4 cm^2 from *P. tinctorum* inhabited the secondary forest. Each transplanted thallus was fixed on 5x5 cm black polyethylene net mesh size 2x2 mm by 0.5 mm monofilament fishing line (Figure 1.). Forty transplanted thalli were fixed on a transplanted frame made from PVC pipe lining with black polyethylene net. They stand on the ground at 45-degree inclination facing the East under 50% black shading net (Figure 1).

The transplanted thalli received extra moisture from vapor evaporated from soil watering and spraying distilled water directly over thalli. They composed of four treatments as show in table 1.

Table 1. The four water treatments on the transplanted lichen *P. tinctorum* conducted in the secondary forest at KYNP.

Thallus	**Soil**	
	Dry soil (SD)	Soil watering (SW)
Dry thallus (TD)	SD-TD	SW-TD
Thallus watering (TW)	SD-TW	SW-TW

These treatments were organized on ten transplanted frames, which divided into each 5 wet soils and 5 dry soils. A transplanted frame had 8x5 thalli (column x row), of which 2 columns alternately treated by TD and TW, (Figure 1), making up (4x5) x 5 x 2 = 200 thalli. In addition, two thalli were transplanted to each 10 transplanted frames for measuring the initial physiological parameters. All together making up a total of 210 transplanted thalli. The experiment lasted from November, 2015 to November, 2016.

Growth and Biomass measurement:Twenty thalli of each treatment from the transplanted frames were randomly selected for growth measurement. The photogrammetry method was used to calculated growth rate according to Hooker and Brown (1977)[5] and Sancho *et al.*, (2011)[6]. Photographs of the thalli on the transplanted frames were taken by a 50 mm lens Canon SH60. Area and diameter were calculated by AxioVision software LE Rel. 4.1 from Carl Zeiss Industrielle Messtechnik GmbH, Germany. Twenty thalli from the transplanted frames were weighed before and after transplantation for biomass measurement. The growth rate and biomass was calculated based on Hill (2002).[7]

Photosynthesis and Chlorophyll fluorescence: Five thallus of each treatment were measured for net photosynthetic rate (P_N) and Chlorophyll fluorescence under 350 □mol $m^{-2}s^{-1}$ light intensity, 100% thallus water content and 25°C room temperature at laboratory of Ramkamhaeng University. P_N was measured by LI6400 (Licor inc. USA) using the conifer chamber. Chlorophyll fluorescence parameter, Fv/Fm, was measured by mini-PAM (Walz inc. Germany) under same condition. Fv/Fm was used to evaluate thallus vitality.

Secondary metabolites analysis: Lichen samples were air-dried at room temperature for two days and ground into powder with liquid nitrogen, and were then sieved through a 500 µm filter. A ten milligram of samples was accurately weighted and extracted for lichen substances by acetone after soaking overnight. The extracts were filtered and evaporated to dryness. The residues were then dissolved by small amount of methanol and diluted to exact volume 70:30 of methanol: DI water using benzoic acid as an internal standard. The solution samples were filtered through 0.45 µm syringe membrane before injection to HPLC.[8]

Data analysis; The differences of growth rate and secondary metabolites among the four treatments (SD-TD, SD-TW, SW-TD and SW-TW) were examined by one-way ANOVA, and all pairwise multiple comparison verified by Tukey Test ($P<0.05$).

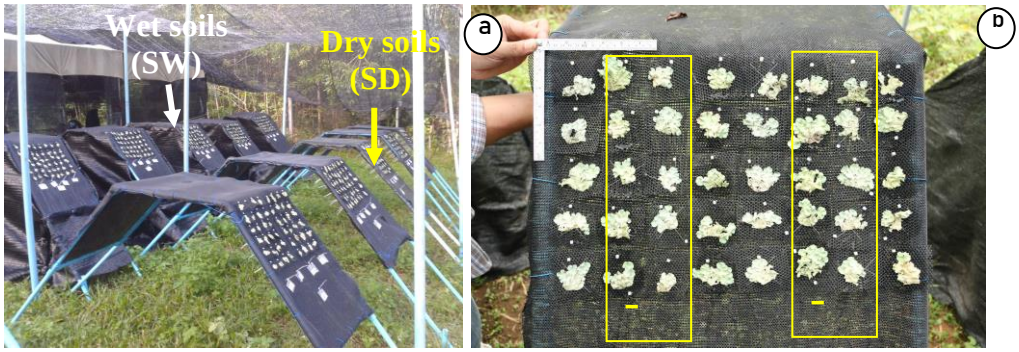

Figure 1. Transplantation of the lichen *P. tinctorum* at KYNP, (a) the transplanted frames stand on the ground at 45-degree inclination facing the East (b) the transplanted thalli on black polyethylene nets were fixed on the transplanted frame. The treatment was: soil watering (SW), without soil watering or dry soil (SD), thalli sprayed with distill water (TW) and thalli without water (TD).

Results and Discussion: Figure 2 demonstrated growth, biomass production, photosynthesis and chlorophyll fluorescence (Fv/Fm) of the transplanted lichens.

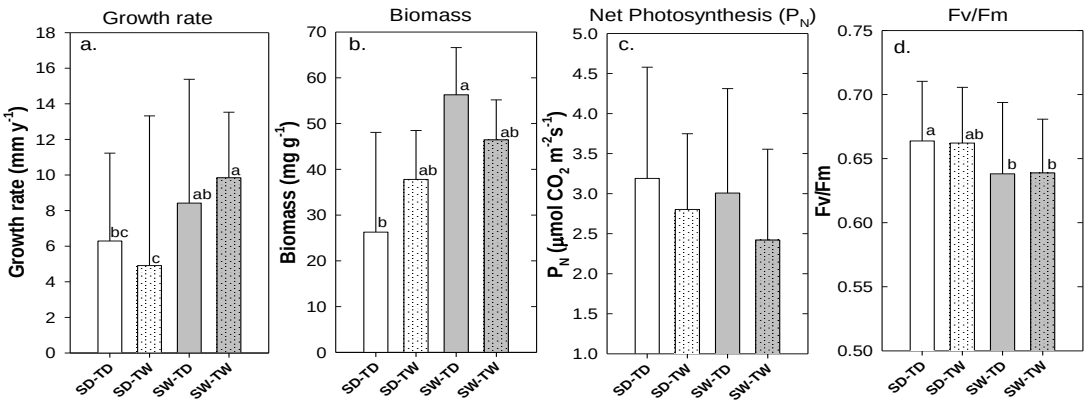

Figure 2. Mean values of growth rate (a), biomass (b), Net photosynthesis (c) and Fv/Fm (d) of lichen *P. tinctorum* after transplantation under SD-TD=control-dry soil without TW, SD-TW =dry soil with TW, SW-TD=Wet soil without TW and SW-TW=Wet soil with TW. Different letters over bars refer to statistically significant difference (Tukey test, $p < 0.05$).

Evaporative moisture from soil watering without thallus spraying (SW-TD) raised up biomass and growth over the thalli in the natural condition, without soil and thallus moistening, (SD-TD). Regardless, net photosynthesis (P_N) and Fv/Fm seem to be lower between such treatments, but at non statistical different (Figure 2).

Thallus spraying (SD-TW) resulted in the higher biomass, but lower growth than the treatment without extra water on thallus (SD-TD). Whilst, P_N and Fv/Fm were not benefited from thallus watering as these values were not significantly different between the two treatments.

Remarkably, by given extra water to the transplanted lichens both through soil watering and direct spraying the thalli (SW-TW) resulted in the highest growth rate among all treatments. Nevertheless, biomass production was lower than the treatment that received only soil watering without thallus wetting (SW-TD).

Most interestingly, we found that soil watering every day without thallus spraying (SW-TD) increased growth, biomass and P_N of the transplanted lichens more than thallus watering (once in two weeks) without soil watering (SD-TW), although Fv/Fm did not follow this pattern.

Watering both soil and thallus enriched growth to the highest rate among all treatments. However, biomass and P_N was lower than soil watering alone, whilst Fv/Fm almost equal to soil watering, and seems to be the lowest among all.

Therefore, soil watering should be used as a basic guideline for future lichen transplantation in order to stimulate lichen production for commercial uses under sustainable utilization of natural resources and conservation policy. Notwithstanding, additional researches should be conducted to applied these information in different environment, along with applying growth stimulating factor, fertilizer and hormone, to achieve the supreme condition for biomass production of lichens.

Secondary metabolites; Lecanoric acid, the major substance of this lichen, in all treatments declined after transplantation (Figure 3a). The highest values were 2357 □g g^{-1} in SW-TD, while the lowest values were recorded in SD-TD (1877□□g g^{-1}).

Atranorin of dry soil treatments (SD-TD and SD-TW) increased slightly. While wet soils (SW-TW and SW-TD) declined as much as 28% of the initial. The highest amount was 185 □g g^{-1} from SD-TW (Figure 3b), with a significantly difference between SW-TW and SW-TD ($p<0.05$).

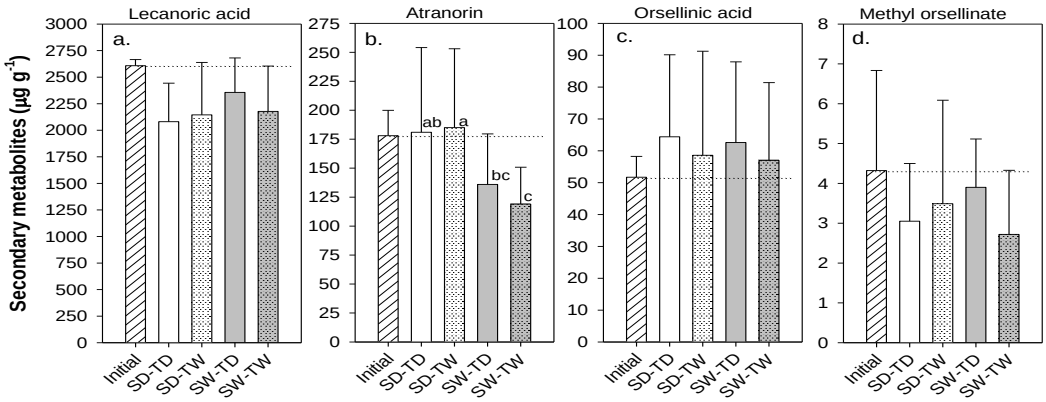

Figure 3. Mean values of the secondary metabolites in the lichen *P. tinctorum* after transplantation in different water treatments. Lecanoric acid (a), Atranorin (b), Orsellinic acid (c) and Methyl orsellinate (d) SD-TD=Dry soil without TW (control), SD-TW=Dry soil with TW, SW-TD=Wet soil without TW and TH, SW-TW=Wet soil with TW. Different letters over bars refer to statistically significant difference (Tukey test, $p < 0.05$).

Orsellinic acid of all treatments increased after transplantation (Figure 3c). Interestingly, dry state thalli (SD-TD and SW-TD) had higher concentration of this metabolite than the wetted thalli (SD-TW and SW-TW), although they were not different statistically.

Methyl orsellinate in all treatments obviously declined more than other lichen substances after transplantation (Figure 3d). Particularly, under the wet condition, SW-TW, the amount reduced as much as 37%. Whilst lichen in dry soil (SD) and wet soil treatments (SW) fell 23 and 31% respectively.

Lichens produce secondary metabolites for protection against environmental stress and defend from biological threatening.[1,9] Overall reduction of secondary metabolites after transplantation on the man-made substrate, the black polyethylene net, could possibly be low stress condition and less threaten by other organisms.

Conclusion:

1. Evaporative moisture from soil watering alone without thallus spraying, elevated growth, biomass and P_N of the transplanted lichens more than only thallus watering.
2. Thallus spraying alone without soil watering, although improved biomass production, but reduced growth of the lichens grew naturally, neither soil nor thallus watering. Whilst, P_N and Fv/Fm were not benefited from thallus watering.
3. Watering both soil and thallus enriched growth to the highest rate among all treatments, although biomass and P_N was lower than soil watering alone.
4. Both water treatments, soil and thallus, although stimulated growth and biomass, but seems to reduce natural products of the lichens. Particularly, lecanoric acid, which is the major metabolite of this lichen. Only orsellinic acid, the minor product, increased as a consequence of water treatments.

References:

1. Rankovic´ B, Studzińska-Sroka E, Fałtynowicz W. 2015; 16:34-43.
2. Phaengphech M, Fuangkaew P, Polyiam W, Watthana S, Boonpargob K. 2016;42: 611-615.
3. Welch AR, Gillman MP, John EA. 2006; 38:177–186.
4. Palmqvist K, Sundberg B. 2000; 23: 1–14.
5. Hooker TN, Brown DH. 1977; 9: 65-75
6. Sancho L, David P, Green TGA, Mercedes V, Pintado A. 2011; 34(6):813-822.
7. Hill DJ. 2002. Protocols in Lichenology; 255-280
8. Thepnuan P, Sriviboon C, Rukachaisirikul T, and Boonpragob K. 2013; 38: 1
9. Deduke C, Timsina B, Piercey-Normore MD. 2012. International Perspectives on Global Environmental Change; 197-230.

Acknowledgements: This study was supported by the National Research Council of Thailand (NRCT). We wish to thank Mr. Sumrit Senglek for their assistance in fieldwork and the officers and staffs at Khao Yai National Park for their kind cooperation. Our gratitude also goes colleagues at Lichen Research Unit, Ramkhamhaeng University.