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PHYSIOLOGICAL STUDY OF *LEMNA MINOR* AS BIOREMEDIATOR UNDER VARIOUS LD CYCLES

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ABSTRACT

Response of *Lemna minor*, to hormones, biomarkers and enzymes towards various Light and Dark cycles was studied in Hogland's nutrient solution. Gibberellic acid 1.0 mg/lit had a remarkable effect in retarding the chlorophyll destruction, where kinetin had no effect on the retention of chlorophyll in any condition used. GA_3 treated plants enhanced the cell division and delayed the senenscence over kinetin which affected the frond multiplication. The effect of darkness is abolished by brief light exposures on biomarkers like frond weights, protein, Chlorophyll content which is clearly showed in catalase and peroxidase activity at various Light-Dark cycles. LD cycles in model A (continuous L&D) showed the adverse effect on chlorophyll contents. Chl b showed a steady decline with decreasing dark periods where as Chl-a is linearly increased. In model B, under treatments of LDCs, the content of Chl-a, Chl-b and Chl-total showed a decline order in comparison with control ($^1/_4$ L / $22^3/_4$D), no matter increasing their fresh weights. Protein content increased in fronds is unaffected after various LD interruptions.

Endogenous rhythms of catalase and peroxidase activities in *Lemna minor* established a free running period of the oscillation between 8 to 17 O'clock. At 2 pm 4A (10L/14D), 4B (1L/$8^{3/4}$D/ ¼

KEYWORDS:
Lemna minor;
Bioremediator;
LD Cycles;
Catalase;
Peroxidase.

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L/14D) , 3C ($\frac{1}{4}$L/ 2D/ $\frac{1}{4}$ L/$2^{1/2}$ D/ $\frac{1}{4}$ L/$2^{1/2}$D/ $\frac{1}{4}$L/1 $\frac{3}{4}$D/1/4D/14 D) LD treatments showed an increasing value of catalase activity, where peroxidase activity is observed more at 2pm in 2A (8L/16D) and 2B (1L/6$\frac{3}{4}$ / $\frac{1}{4}$ L/16D) conditions but 3C at 5 pm. Catalase activity suppressed the peroxidase activity at 8am, but reversed at 2pm and 5pm. Peroxidase showed the highest amplitude value under various LD cycles. Pigment analysis resulted that *Lemna minor* does not require photosynthesis but need highly nutritional situations for growth and can be used as a potential source of protein and a natural source of bioremediator.

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INTRODUCTION

Lemna minor, a short day duckweed; is a small green, seed bearing aquatic perennial plant multiply rapidly by vegetative division. The leaves float freely on surface of a water body while the root hangs below the surface to obtain nutrients from the water. Duckweed is an excellent plant model for biochemical (Sherameti et. al. 2002) and physiological studies (Krajncic and Devide 1980, Chi et. al 2012). It can survive and recover from extremes of temperature (7^oC – 35^oC), nutrient loading, nutrient balance and pH (4.5-9.0). Plant growth is dependant not only on the composition of nutrient media but also on external conditions such as temperature, photoperiod and light quality. Many daily rhythms of biological activity persist, sometimes indefinitely when organisms are kept in constant temperature and continuous light conditions. The rhythmi city exhibited by organisms in their activity is truly an endogenous one as biological clock. This experiment was conducted in Hogland's nutrient solution with *Lemna minor* L. and studied its response to hormones, biomarkers and enzymes towords various L D cycles. Plants have endogenous biological clocks known as circadian rhythms that allow organisms to anticipate and prepare for daily and seasonal changes for their survivelity in

environments (Inoue et. al 2018). Circadian clock can be entrained by environmental factors such as light, temperature and nutrient status to synchronize internal biological rhythms with surrounding environments and regulate various physiological, developmental and reproductive processes (Mc Clung 2006; Mc Clung, 2011). Phase shifting (Phase response curve), a decisive factor for synchronization with external 24 h cycle is in response of various phases to light and temperature. Details of these differences become clear by applying pulses of light in DD (total darkness) or of higher light intensity in week LL (continuous illumination) or pulses of higher temperature in lower temperatures. If the time of environmental interruption is plotted against the phase shift (measured in terms of advances or delays compared to control) then the phase response curve (PRC) for the rhythm is obtained. PRC is a much used concept in rhythm research because it provides an identifiable profile of the under laying unseen oscillator that controls the overt measured rhythm. An organism which has been exhibiting a specific rhythm in response to a natural exposure to light during the day time and darkness at night may have its rhythm rephased by 12 hours through exposure to light at night and darkness in the day time. These rhythms are innate and inheritable. In many cases a single stimulus of light is necessary to evoke the oscillation. Endogenous rhythms are involved in photoperiodic response that exposure of organisms to cycles with very long dark periods, and interruption of this dark periods at different points with brief periods of illumination and another exposure of organisms to cycles of unnatural length, ranging from very short to very long cycles, each cycle length being determined primarily by the length of the dark period. In this plant a large effect of darkness during the light period is studied. Rhythmic response of light and phytohermones is essentially evaluated from $22^{3}/_{4}$L to 12hr alterations include long periods of darkness in the light periods with brief light interruptions towards affecting change in growth pigment, enzymatic activities and protein content under physiological clock. *Lemna minor* a typical short day plant, the exact shortest day length at which flowering is entirely suppressed varies slightly but is roughly 14 hours. However this plant can be grown in nutrient rich medium and does not require photosynthesis for growth.

RESEARCH METHODS

Common Duckweed (*Lemna minor*) was maintained aseptically in 250 ml Erlenmeyer flask containing 50 ml of Hoagland's medium in each flask. Hoagland's medium was prepared as per standard procedure (Hoagland 1975). Plants were cultured under LD cycles as per the schematic model (A, B, and C) of the experiment under laboratory conditions at 27 ± 1^0C. Light and dark treatment was given from artificial light sources of three 15 watt Sylvania fluorescent lamp which provide 235 ft-c ($7.4x10^3$ergs.cm^{-2} Sec^{-1}) at a distance of 48.4 cd from flasks.

Phytoassay sensitivity of *Lemna minor* was assessed in different photoperiods with growth promoting hormones like Gibberelin and Kinetin at different concentrations of 1mgL^{-1}, 0.1mgL^{-1} and 0.01mgL^{-1}. Growth was estimated by chlorophyll contents (Chl-a, Chl-b and Chl-total), protein, catalase and peroxidase from the fresh weight of 15 fronds as the measured parameter. Chlorophyll was estimated from fresh leaf material by standard procedure of Arnon (1949). Protein was estimated by the procedure described by Lowry et. al (1951) using Bovin serum albumin as standard. Both Catalase and Peroxidase was estimated from freshly weighed 15 fronds samples of *Lemna minor* using the procedure of Maehly and Chance, 1967.

RESULTS

Effect of hormone treatment:

Effects of GA3 and kinetin separately on growth of *Lemna minor* is presented in table-1. GA_3 retards chlorophyll destruction in comparison with their fresh weight to that of the control. Optimal concentration of GA_3 for growth was found to be 1 mgL^{-1}. Concentration of 0.1mgL^{-1} has a smaller effect which still lowers one are in-effective. Whereas kinetin did not influence the chlorophyll content at any concentration in comparable with fresh and dry weights of control and GA_3 treated plants; but it enhanced the cell division. Therefore, the cultures with GA_3 attain the green color of the chlorophyll that delayed senescence and kinetin affected the frond multiplication (Table-1)

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Table 1: Chlorophyll and protein content in fronds of *Lemna minor* as influenced by gibberllic acid and kinetin.

Treatments	Fresh Wt. (mg/15F)	Dry Wt. (mg/15F)	Protein ($mg.g^{-1}$ FWt.)	Chlorophyll content ($mg.g^{-1}$ FWt)		
				Chl-a	Chl-b	Total Chl.
Control	250	60.5	2.8	1.4	0.3	1.7
GA_3 1mg/lit	335	75.5	5.3	1.7	0.5	2.2
K 1mg/lit	305	69.8	2.7	1.3	0.4	1.7
GA_3 0.1mg/lit	310	70.8	4.3	1.6	0.4	2.0
K 0.1mg/lit	240	58.6	2.2	1.2	0.2	1.4
GA_3 0.01mg/lit	220	58.6	4.1	1.4	0.3	1.7
K 0.01mg/lit	190	48.4	2.2	1.1	0.2	1.3

Effect of alternate light-dark cycles on pigment contents:

The formation of the pigment is determined by the general light and dark reactions and the structure of green pigments is due to the kind of plant and the structure of chloroplast. On the basis of the change in the pigment quantity, the duckweeds were the most sensitive to the light and dark treatments (Table-2). In the effect of the LD Cycles, there are certain general tendencies, which are only modified in a small degree by different degrees of stability. Compare with the control, various light and dark conditions showed adverse effect on chlorophyll contents. The pigment chlorophyll-a showed a steady decline with decreasing dark periods whereas chlorophyll-b pigments are linearly increased. This is also evident from chlorophyll-total. As a result of linearly decreasing dark photoperiods, the quantity of the chlorophylls decreases; particularly the decomposition of chlorophyll-a is considerable. In the model 'B' under treatment of LD Cycles, the content of chl-a is more than the chl-b according to their fresh weight. But the pigments chl-a, chl-b and the total chlorophyll content showed a decline order in comparison with the control, with no mater of increasing their corresponding fresh weights undergoing ¼ L interruptions. In case of model 'C' the treatments 2 C and 3 C with the frequent light dark interruptions, there is a decline in the chlorophyll content to that of the control (Table-2).

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Table 2: Effect of various photoperiods on fresh weight, chlorophyll, protein and its rhythmicity in *Lemna minor*.

LD Cycles		Treatments	F.Wt.15 F (mg)	Protein (mg/g fwt)	Chlorophyll content(mg/g fwt)		
					Chl-a	Chl-b	Total Chl.
A	1	Control (1 $\frac{1}{4}$L/22$\frac{3}{4}$D)	266.60	2.16	1.08	0.38	1.46
	2	8L/16D	276.60	2.46	1.09	0.27	1.36
	3	9L/15D	281.33	3.66	1.07	0.14	1.21
	4	10L/14D	278.33	3.15	1.05	0.24	1.29
	5	11L/13D	280.00	3.23	1.12	0.20	1.32
	6	12L/12D	275.66	3.70	1.11	0.10	1.21
B	1	Control (1 $\frac{1}{4}$L/22$\frac{3}{4}$D)	266.60	2.16	1.08	0.38	1.46
	2	1L/6$\frac{3}{4}$D/$\frac{1}{4}$L/16D	274.00	2.26	1.69	0.15	1.84
	3	1L/7$\frac{3}{4}$D/$\frac{1}{4}$L/15D	274.33	3.43	1.05	0.14	1.19
	4	1L/8$\frac{3}{4}$D/$\frac{1}{4}$L/14D	271.00	2.83	1.04	0.13	1.17
	5	1L/9$\frac{3}{4}$D/$\frac{1}{4}$L/13D	270.00	1.98	1.07	0.15	1.22
	6	1L/10$\frac{3}{4}$D/$\frac{1}{4}$L/12D	276.60	3.03	1.07	0.16	1.23
C	1	Control (1 $\frac{1}{4}$L/22$\frac{3}{4}$D)	266.60	2.16	1.08	0.38	1.46
	2	1L/8$\frac{3}{4}$D/$\frac{1}{4}$L/14D	271.33	2.56	1.06	0.15	1.21
	3	$\frac{1}{4}$L/2D/$\frac{1}{4}$L/2D/$\frac{1}{4}$L/2$\frac{1}{2}$D/ $\frac{1}{4}$L/1 $\frac{1}{4}$D/$\frac{1}{4}$L/14D	274.66	3.10	1.06	0.16	1.12

Changes of protein contents in the leaves under various light-dark cycles:

Both control and treatments did not affect the proteins. The content of the protein in *Lemna minor* leaves increased with increasing respective fresh weights without affected by various light-dark interruptions. (Table-2)

Effect of light-dark cycles on the enzyme activity:

Changes in the catalase and peroxidase activity of *Lemna minor* plants grown in the nutrient medium under various light and dark conditions at 8 am, 2 pm and 5 pm day times. (Table-3, Fig.1, Fig.2). At 8 am the catalase activity is less to that of the control and increasing at 2 pm but gradually decreased at 5 pm. In 4A (10L/14D) condition catalase activity is more in (121.87); in 4 B condition(1L/8 3/4D/1/4L/14D) is 129.24 and in 3 C conditions is 112.50 at 2 pm (Fig.1). The peroxidase activity is also enhanced at 2 pm than 8 am and 5pm. In the 2A(8L/16D) condition showed the highest of 176.16 peroxidase activity and in 2B condition of light-dark the peroxidase activity is more of 122.21 at 2 pm. Where as in 3C conditions, peroxidase activity is more of 121.53 at 5 pm. shown (Table-3, Fig.2). Therefore it has shown that the catalase activity suppressed the peroxidase activity at 8 am and reversed effect at 2 pm and 5 pm.

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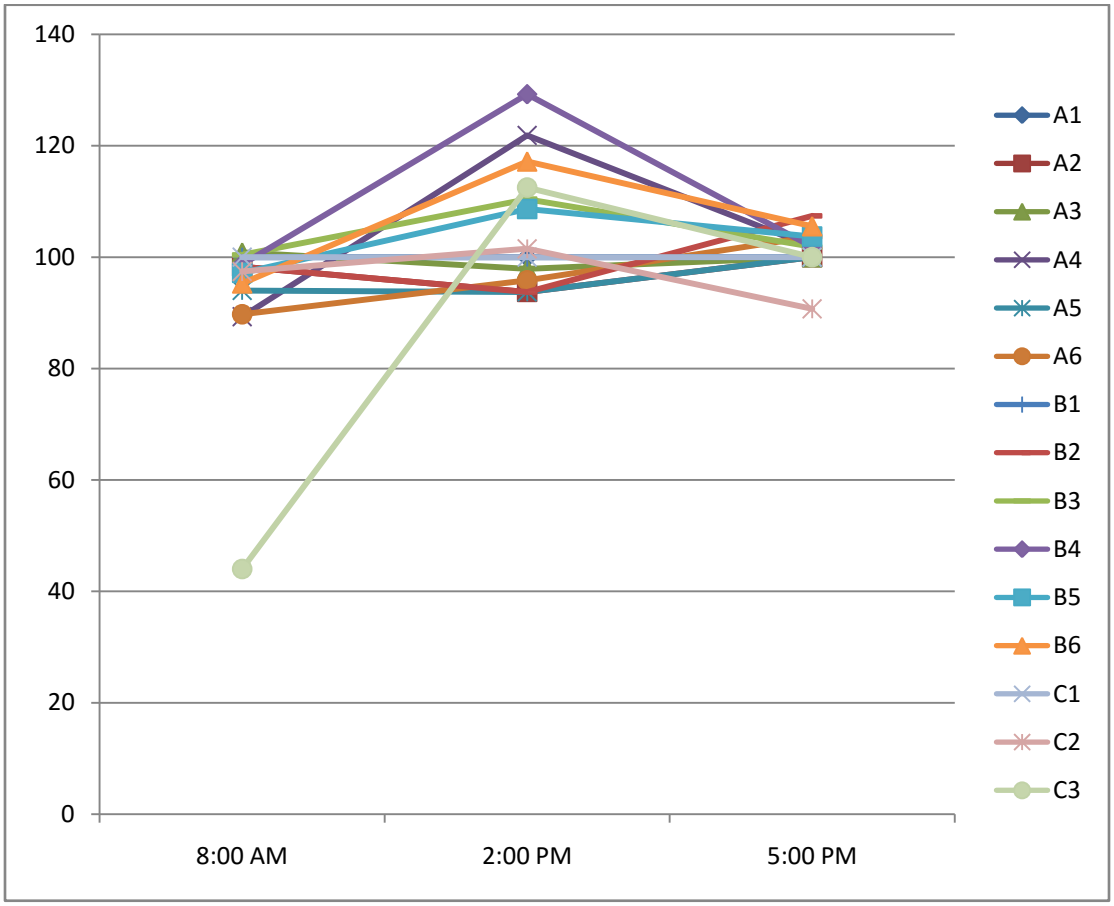
Figure 1: Rhythms of Catalase activity of *Lemna minor*.



Perioxidase activity showed the high amplitude value than the catalase activity under the treatments of various light-dark conditions. In the treatments A, B and C types the amplitude of peroxidase activity was enhancing in B type treatment of 1 hL and1/4 hL light interruptions respectively with the amplitude of 34.34 was highest in the treatment 4B(1L/8 3/4D/1/4L/14D). Catalase activity showed the highest amplitude value 1.36 under 4A(10L/14D) conditions from A,B,C conditions of photoperiods (Table-4).

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Table 3: Effect of photoperiods on catalase and peroxidase activity and its rhythmicity in *L. minor*.

LD Cycles		Treatments	Activity at 8 AM (%)		Activity at 2 PM (%)		Activity at 5 PM (%)	
			Catalase	Peroxidase	Catalase	Peroxidase	Catalase	Peroxidase
A	1	Control ($1\frac{1}{4}L/22\frac{3}{4}D$)	100.00	100.00	100.00	100.00	100.00	100.00
	2	8L/16D	98.28	34.42	93.75	176.19	100.00	171.86
	3	9L/15D	100.85	34.86	97.91	156.94	99.99	140.21
	4	10L/14D	89.31	34.82	121.87	130.48	101.85	114.47
	5	11L/13D	94.01	25.71	93.75	156.82	99.99	115.53
	6	12L/12D	89.74	45.03	95.83	174.43	103.70	83.10
B	1	Control ($1\frac{1}{4}L/22\frac{3}{4}D$)	100	100	100	100	100	100
	2	$1L/6\frac{3}{4}D/\frac{1}{4}L/16D$	98.28	14.06	93.75	122.21	107.40	33.61
	3	$1L/7\frac{3}{4}D/\frac{1}{4}L/15D$	100.42	9.13	110.41	95.98	101.85	29.25
	4	$1L/8\frac{3}{4}D/\frac{1}{4}L/14D$	98.71	4.06	129.24	139.46	101.85	25.59
	5	$1L/9\frac{3}{4}D/\frac{1}{4}L/13D$	97.19	19.36	108.64	108.64	103.70	38.74
	6	$1L/10\frac{3}{4}D/\frac{1}{4}L/12D$	95.29	6.40	117.17	117.38	105.55	62.44
C	1	Control ($1\frac{1}{4}L/22\frac{3}{4}D$)	100	100	100	100	100	100
	2	$1L/8\frac{3}{4}D/\frac{1}{4}L/14D$	97.43	59.09	101.51	101.51	90.73	121.53
	3	$\frac{1}{4}L/2D/\frac{1}{4}L/2D/\frac{1}{4}L/2\frac{1}{2}D/\frac{1}{4}L/1\frac{1}{4}D/\frac{1}{4}L/14D$	44.01	34.52	112.50	112.50	100.00	8.22

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Figure 2: Rhythm of Peroxidase activity of *Lemna minor*.

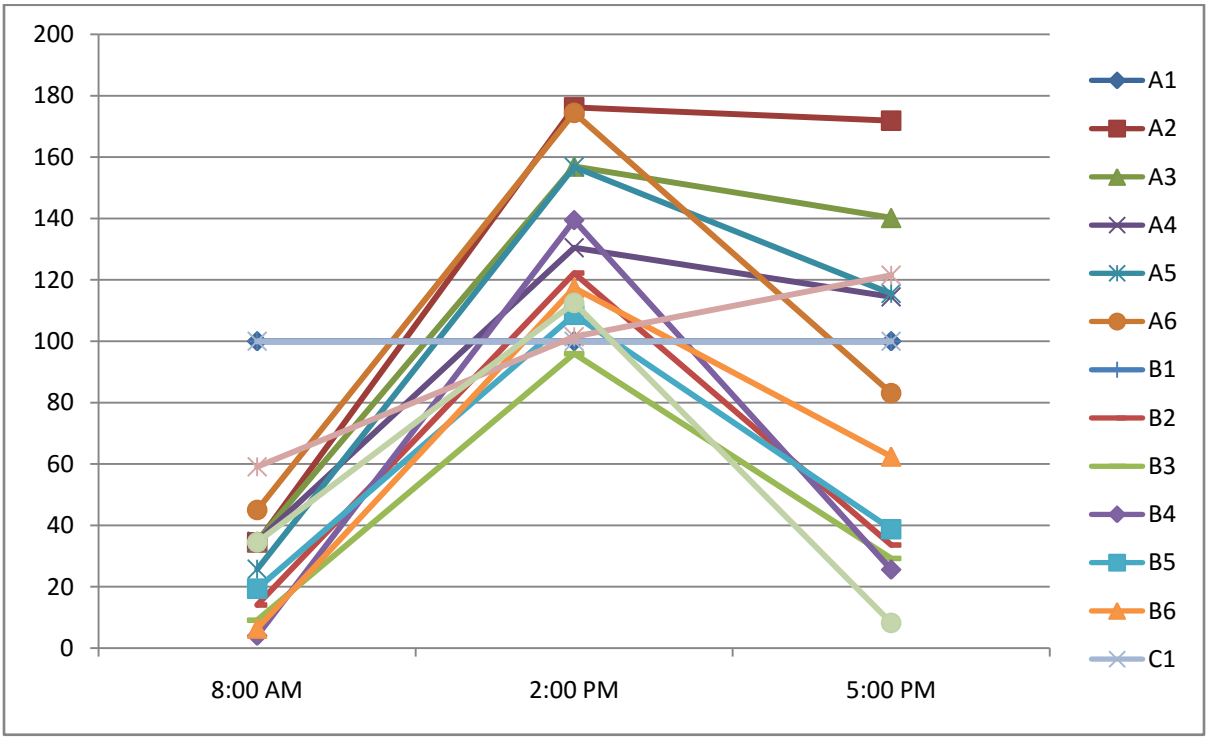

Table 4: Rhythm (Amplitude) for Catalase & Peroxidase activities of *Lemna minor* under various LD Cycles

LD Cycles		Treatments	Amplitude	
			Catalase Activity	Peroxidase Activity
A	1	Control ($1\frac{1}{4}$L/$22\frac{3}{4}$D)	1.00	1.00
	2	8L/16D	1.06	5.11
	3	9L/15D	1.03	4.50
	4	10L/14D	1.36	3.74
	5	11L/13D	1.06	6.09
	6	12L/12D	1.15	3.87
B	1	Control ($1\frac{1}{4}$L/$22\frac{3}{4}$D)	1.00	1.00

	2	1L/6$\frac{3}{4}$D/$\frac{1}{4}$L/16D	1.14	8.69
	3	1L/7$\frac{3}{4}$D/$\frac{1}{4}$L/15D	1.09	10.51
	4	1L/8$\frac{3}{4}$D/$\frac{1}{4}$L/14D	1.30	34.34
	5	1L/9$\frac{3}{4}$D/$\frac{1}{4}$L/13D	1.11	5.61
	6	1L/10$\frac{3}{4}$D/$\frac{1}{4}$L/12D	1.22	18.34
C	1	Control (1 $\frac{1}{4}$L/22$\frac{3}{4}$D)	1.00	1.00
	2	1L/8$\frac{3}{4}$D/$\frac{1}{4}$L/14D	1.11	2.05
	3	$\frac{1}{4}$L/2D/$\frac{1}{4}$L/2D/$\frac{1}{4}$L/2$\frac{1}{2}$D/ $\frac{1}{4}$L/1 $\frac{1}{4}$D/$\frac{1}{4}$L/14D	2.55	3.25

DISCUSSION

Rhythmicity remains one of the characteristics of life and express itself at all levels of organization (Bu nning, 1973). Combining these rhythms with time measurement was considered as physiological clock. This is an oscillation in a biochemical, physiological or behavioral function which continues to oscillate under constant but permissive conditions of light and temperature with a period of approximately but not exactly 24 h (Sweeney, 1976). Entrainment of circadian rhythms establishes a definite phase relation between the innate oscillation of the organism and periodicity of the environmental change. This plant can be grown with sucrose as a substrate and this does not require photosynthesis for growth. In some cases cytokinin was shown to enhance synthesis of certain enzymes (Borries, 1977) or to induce synthesis of protein (Karavaiko et. al 1978). The activity of peroxidase in plants is controlled by set of hormonal and environmental factors (Galston and Davis, 1969).

Plant growth regulator compounds including plant hormones, profoundly influence the growth and differentiation of plant cells, tissues and organs (Utami et. al 2018). Once synchronized with environment, the circadian clock can anticipate daily environmental fluctuations, this coordinating diverse physiological and developmental processes in a time of day specific manner which enhances adaptability (Green et. al 2002, Michael et. al 2003, Dodd

et. al 2005, Yerushalmi et. al 2011,Valentin et. al 2019) by modulating hormonal pathways (Robertson et. al 2009). Majewsky et. al 2014 was confirmed that growth was increased by GA_3. Oota and Tsudzuki 1971, reported that the number of fronds was increased to 125% by the application of 100µM GA_3 and the chlorophyll content was increased after treatment with 5µM GA_3. Gibberellic acid in concentration of 1.0 mgL^{-1} had a remarkable effect in preventing the chlorophyll destruction. So that the *Lemna minor* remained green for longer days than the control ones. It may be concluded that the effect of hormones is primarily in the frond multiplication and therefore specific for prevention of chlorophyll destruction in *Lemna minor*. Kinetin (as cytokinin in plants) had no effect on the relation of chlorophyll in any concentration used (Table 1). This may regulate cell division, shoot and root growth and leaf senescence (Werner and Schmulling, 2009, Kieber and Schaller 2014). Cytokinin causes phase delays of 1 to 3 h affects growth (Hanano et. al 2006 and Zheng et. al 2006), which is observed in terms of fresh weight, dry weight, chlorophyll content and protein factor of the fronds (Table 1). However, circadian periodicity is not very strongly affected by cytokinin (Hanano et al., 2006; Salomé et al., 2006; Zheng et al., 2006). These reports suggested that the influence of cytokinin on LD functions is rather moderate. Recently cytokinin was also shown to play various roles in response to adverse environmental conditions (Ha et. al 2012; O'Brien and Benkova, 2013) which may enhance plant fitness and survival (Yerushalmi et. al 2011).

In *Lemna minor* plants a large effect of darkness during the light period is recorded (Table 2-4). The results clearly indicated that the effect of darkness is abolished by a few brief light exposes and that it is evidently controlled with a genuine effect of timing and not with the total amount of light is also in agreement with the findings of Hillman, 1963. Catalase and peroxidase extracted from *Lemna minor* entrained in various LD cycles displayed circadian rhythmicity. Both enzymes are main antioxidizing enzymes, as one of the main factors that maintain redox hemiostatis (Muranaka et al.2015). Although the results from the enzyme analysis showed that the free running did deviate from exactly 24 hr in all the 15 LD treatments were expressed as percentage of control. Results from this study with *Lemna minor* provide yet another property in support of the rhythm patterns of these two enzyme activation as very similar

and essentially differed only in the levels of the various LD cycles (Table -3, Fig. 1 & 2). The findings of the experiments support circadian rhythmicity in catalase and peroxidase activities in *emna. minor* and establishes the free running period of the oscillations between 8 to 17 O' clock i.e. 9 hrs (Table-4). This investigation supports the Bunning's hypothesis (1982) that endogenous rhythmicity changes according to timing of light conditions and thus in performing a timing in enzyme activities and on amplitude range indicating "Physiological Clock". These studies expressed that there is a marked differences between chlorophyll and protein content (Table-2) with that of the enzyme rhythmic activity (CAT & POD).

Analysis of chlorophyll content from Table-2, justified that *Lemna minor* does not require photosynthesis rather need a nutritious medium for its growth. It might be suggested as the entire plant body consists of metabolically active non-structural tissue (Wolverton and Mc Donald, 1980). Duckweed growth on water with relatively high levels of N, P, K which concent rates the minerals and synthesizes protein. A direct relationship was found between crude protein content of duckweed and the nitrogen content of the culture system which is anticipated with the findings from frond weight and protein content at respective LD rhythms. The growth of duckweed is largely a function of environmental temperature and light, nutrient status of the culture media and the degree of growth of the plants (Hassan and Chakrabarti 2009). Certainly, the growth rate of duckweed clones in different naturals (Rejmankova,1975) and laboratory (Landolt ,1957) conditions varied.

These experiments underlined *Lemna minor* also influenced by endogenous rhythmicity due to environmental responses. The circadian clock entrained by environmental cues such as light, temperature and nutrient status through multiple input pathway (Inoue et.al 2017). Then the clock regulates various biological processes at an appropriate frame of day through output pathways.

CONCLUSION

A circadian endogenous rhythm persists in *Lemna minor*, a common duckweed plant mostly sensitive to light, high temperature and highly nutritive decayed water for growth and enhances its survival. It showed a fitness and level of adaptation, and requires highly mineral status for vegetative multiplication rapidly rather depending upon photosynthesis. So *Lemna minor* can be used as a natural source of bioremediator for waste water bodies for commercial purposes.

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