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Impact of Light Emitting Diode Spectrum on Symbiotic Association and Antioxidant Capacity of Fern *Azolla caroliniana* Grown in an Invitro System

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Abstract

Azolla is as fastest growing aquatic fern widely found in stagnant water bodies and have lots of research and economical value due to its symbiotic association with cyanobacteria. The purpose of this research was to observe the effect LEDs red, blue and white light as control on growth rate, antioxidant capacity and symbiotic association of Azolla *caroliniana*. Under the experiment light intensity was standardized with 58 µmol m^{-2} s^{-1}. The results showed that red light shows the highest effect on growth rate of fern. The antioxidant activity was measured by the radical scavenging test (DPPH), Ascorbate Peroxidase (APx) and Superoxidase dismutase (SOD). DPPH and Ascorbate Peroxidase (APx) showed the highest activity under the red-light treatment therefore utilized in pharmacological application. Apart, the research was also carried out to study the effect of light on symbiotic association and ultrastructure of fern. Based on confocal Imaging analysis data, it is revealed that exposure to red and blue light stimulates the formation of pectin projections or teat cells. The possible role of the projections in the symbiotic relationship between *Anabaena azollae* provide additional evidence for providing defense role. The present result suggests that among all the light treatment, red light was most beneficial for growth and antioxidant capacity of fern.

***Key words*:** *Azolla caroliniana,* Antioxidant capacity, Cyanobacteria, Monochromatic light, Pore cavity, Symbiotic association

Azolla is considered an important medicinal and economical pteridophyte. Free-floating fern naturally occurs all over the world in warm and temperate region with optimum temperature range between 15-35°C, this plant belongs to the family; Salvaniaceae, order; Salviniales [10]. Out of the total eight species and forty extinct species identified, *Azolla caroliniana* is one of the important ferns that can double its biomass within 3-4 days and have lots of nutritional value [31-32]. The small fern is triangular in shape measuring up to 2.5 cm to 3.0 cm in length and 1-2 cm wide, they are green, blue green and dark red in color coated with tiny hairs which gives them velvet appearance make water repellent [2]. The *Azolla* sporophyte composed of a primary rhizome that splits into lateral roots, all of which produce minute leaflets oriented sequentially, at nodes on the ventral surfaces of the rhizomes, elongated lateral roots descend into the water and absorb nutrient. Each leaf is made up of two lobes, an achlorophyllous dorsal branch and cup-shaped lateral lobe that is airborne and an inert, that provides buoyancy [29]. Reproduction in fern observed by both sexual and vegetative mode. vegetative method through fragmenting of the abscission layer, which is found at the base of each branch. Sexual reproduction is uncommon and appears to be controlled by the male and female gametes.

Recently ferns have perceived more attention towards scientific community because of their significant properties, phytoremediation, livestock feed, biofuel, and medicinal among all, the important property of fern exhibits symbiotic relationships with endophytic blue-green algae named *Azolla-anabaena*, facilitating a positive environment for the cyanobacteria as well as supplying the plant with nitrogen and source of energy in furthermore, these bacteria is crucial for the plant's nitrogen fixation and has commercial value as biofertilizer all across the world [16]. The symbiosis relationship between cyanobacteria and *azollae* is permanent, which means that the two organisms are linked at all phases of the pteridophyte life cycle, and it will pass from one generation to another generation as akinetes inoculate despite whether reproduction is sexual or asexual [27]. A successful co-evolved system can be said to exist because cyanobacteria are present all over the fern's life cycle, supporting its symbiotic character and indicating to a parallel phylogenetic evolution between both the partner, due to its significant connection, the fern is regarded as a "Superorganism".

Light is a form of electromagnetic radiation that can possess range of wavelength ranges from gamma rays to radio waves expressed in meters. The wavelength ranges of the LEDs from red (620-700 nm), blue (400-490 nm), (280-380 nm), and less-efficient photosynthetic colors like cyan (490-520 nm), green (520-570 nm), yellow (570-590 nm), orange (590-620

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nm), and far-red (620-620 nm) (700–800 nm [7]. The spectrum quality of light irradiance, intensity, and photoperiod imparts the effect on anatomical, physiological, biochemical pathway and antioxidant capacity of plants, with therapeutic qualities. LEDs, or light-emitting diodes have shown the promising effect in recent years for application in invitro propagation and crop development. LEDs have advantage over the conventional light source reduced heat radiation, longer durability, and significantly lower power consumption [7].

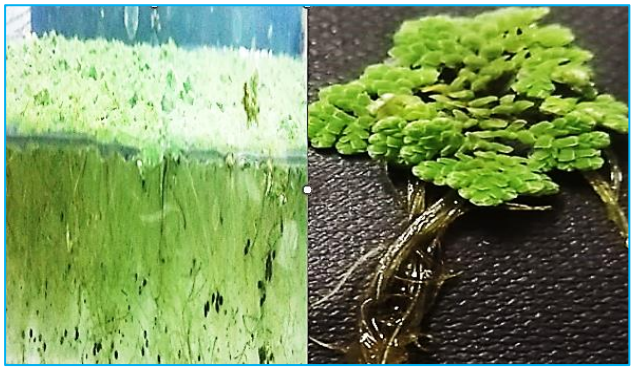

Fig 1 *Azolla Caroliniana* fern collected from the pond

Recently, several studies are conducted to increase the biological activity of plants using LED light. Red and blue light are majorly used as experimental light and have been reported as beneficial effect on the growth and antioxidant properties of plants. Keeping in the view of above account the present publication includes role of different monochromatic light quality and quantity on antioxidant and symbiotic association of fern *Azolla caroliniana*. The effect of LED (red and blue) on leaf pore ultrastructure and symbiotic association are scarcely studied and reported first time. Current study was done to assay the effect of monochromatic red and blue light on *Azolla caroliniana* vegetative growth and assessment of antioxidant capacity, production of important secondary metabolites, and symbiotic association.

MATERIALS AND METHODS

Sample collection and cultivation

The wild fern *Azolla caroliniana* sample was collected from the Badgonda forest in the Mhow Region of Madhya Pradesh and maintained under laboratory conditions. The sample collected was immediately surface sterilized using a 0.1% hypochlorite and 70% alcohol solution for 1 minute and followed by washing with double distilled water to wash unwanted debris and epiphytic bacteria [6]. The sterilized fern was then inoculated in nitrogen-free Espinase and Watanabe medium (1976), with macro and micronutrient. The pH of the medium was maintained at 6.5 to 7.5 with 1 N NaOH/1 N HCl.

Experimental design

LED chambers of specific wavelengths blue, red, and white at 58 μ mol m^{-2} s^{-1} created for experiment. The culture is maintained under photoperiod (16hr:12 hr.) light and dark photoperiod at a temperature of 20–25 °C for 20 days of the cycle. Plants accurately weighing about 10 gm were transferred into transparent plastic trays containing medium in triplicates and placed in a culture room illuminated with different monochromatic lights and observations were recorded for different growth parameter.

Doubling time of Azolla caroliniana

The doubling time of the plant was calculated as the duration in days needed for the biomass to multiply and was calculated by using the formula given by [5].

$$dt = \frac{ln2}{\mu} = \frac{0.693}{\mu}$$

Enzymatic antioxidant capacity assay of Azolla caroliniana

DPPH radical scavenging activity assay

Fern antioxidant and free radical scavenging activity was calculated by DPPH and ascorbate assay in this study. For estimation diphenyl picryl hydrazyl (DPPH) followed protocol given by [14] with slight modification. *Azolla* leaf extract of 1 ml was added to one milliliter 0.1 mM DPPH solution prepared in methanol. The presence of antioxidant activity was examined by change in color form violet/ to yellow color. The tube was then covered with aluminum foil and incubated at 37°C for 30 min and reading was recorded at 517 nm.

DPPH activity (%) = (abs $_{control}$ - abs s_{ample}) / abs $_{control}$ × 100

Ascorbate peroxidase (apx) activity

The enzyme ascorbate peroxidase (APx) is a widely present in plant, animal and bacteria. It is unique to other peroxidases enzyme to scavenge and reduce reactive oxygen species (ROS), such as hydrogen peroxide to water. APx activity was measured using the method given by [22]. Plant was grinded in potassium phosphate buffer and centrifuge at 11,180 g for 15 min at 4℃ .Supernatant was collected and used as crude enzyme extract. Reaction mixture quantity 0.9ml was prepared containing 50 milli molar phosphate buffer, 0.5 milli molar ascorbic acid, 1.0 milli molar H_2O_2, and 0.1 milli molar EDTA. Then add 0.1ml of plant crude enzyme extract in reaction buffer. To initiate the reaction, H_2O_2 was added last and the decrease in absorbance was recorded for 3 min at absorbance 290 nm.

The inhibition % of APx = (abs control - abs sample) / abs control × 100

Super peroxide scavenging activity

The invitro *Azolla caroliniana* SOD antioxidant activity was carried by using photo reduction of NTB (nitro blue tetrazolium) scavenging assay given by [18] with slight modification. Plant was grinded with 50 millimolar ice cold potassium phosphate buffer and centrifuged at 4℃ on 11,180 g for 15 minutes, supernatant obtained was used as crude enzyme extract. Reaction mixture containing, 500μL of NBT, 1.3 mL of sodium carbonate buffer and 100μl of Triton X-100. To initiate the reaction 100 ul of hydroxylamine hydrochloride is added in reaction mixture, using blank without enzyme and NBT. Now incubate reaction at room temperature for 2min and add 70 μl crude enzyme extract, immediately take the reading at absorbance on 560 nm at every 15 s for 1–2 min. The inhibition percentage rate of NBT was calculated as follows:

% The inhibition % of SOD = (abs $_{control}$ - abs $_{sample}$) / abs $_{control}$ × 100

Catalase scavenging activity

Catalase activity was assayed using the method given by [15]. The invitro catalase activity assay of *Azolla* was carried by recording in decline of catalase scavenging activity spectrophotometrically. Fern was grinded with ice cold potassium phosphate buffer and centrifuged at 4℃ for 15 min on 11,180 g. Supernatant collected and followed by adding 30 mM H_2O_2 and make final volume to 3 ml. Catalase activity was tested as the rate of breakdown of H2O2 causes change in absorbance rate per minute as protocol given by [15]. Catalase activity was calculated by formula given:

U/mg = $(Ao - A\ 180) \times \mathrm{Vt}/\mathrm{\mathcal{E}}\ 240\ \times d \times Vs \times Ct \times 0.001$

Where;

$(Ao - A\ 180)$ initial and final absorbance
Vt is total reaction volume (3 mL).
Ɛ 240 is the molar extinction coefficient for H_2O_2 at OD240 (34.9 mol^{-1} cm^{-1})
d is cuvette optical path length (1 cm). Vs is sample volume (1 mL). Ct is concentration of protein in the sample. At 240 nm OD the absorbance change generated by 1 U of enzyme per minute is 0.001.

Visualizing Azolla caroliniana symbiosis and leaf pore cavity
Sample preparation and analysis

Wild species of *Azolla caroliniana* sample were washed with sterile distilled water to remove the debris and epiphytic bacteria. Subsequently fern was surface disinfected with a 0.1% hypochlorite solution. The disinfected plant was grown in experiment chamber and illuminated with monochromatic light of various wavelengths, including blue, red, and white, at a steady light intensity, as quantified by a light meter, at 58 µmol m^{-2} s^{-1} 16:12-hour light dark. For comparison, a culture was also kept under natural condition as control. The culture was maintained under this setup for 20 days. Excised leaves of exposed plant of equal length (5 mm) are washed with distilled water and placed on the slide and processed with coverslips sample analyzed for auto fluorescence on confocal scanning optical microscope (Zeiss LSM510META) at UGC-DAE CSR, Indore.

Image analysis by confocal laser scanning microscopy

Here, in experiment we perform confocal laser scanning microscopy to observe the role of monochromatic light focusing on red, white and blue light on *Azolla* symbiosis and leaf pore cavity organization and structure throughout its developmental course and to better understand how they influence the symbiosis. Autofluorescence was measured at 448 and 505 nm excitation and emission wavelength. This technology helps us to observe the morphological depth of plant surface with thin sample and minimal interference [11]. Method can be employed for their detection of natural pigment in any sample by taking use of the fluorescence generated by the chlorophyll and phycobilin protein of cyanobacteria at 665 nm emission wavelength. Confocal microscopy can be used to detect natural pigment fluorescence in cyanobacteria which has emission wavelength of 665nm (phycobilin and chlorophyll-a) [34]. Under the experiment 10x magnification used for image analysis and looking inside the *Azolla* leaf cavity. cyanobacteria native fluorescence phycobilisomes protein used to image the scanning microscopy. The wavelength 590-nm-long pass filter used to measure the cyanobacterial natural fluorescence's emission wavelengths. At various depths, images of leaf were taken, in order to observe the image, the internal colonization, group of images were taken up to a depth of 50–200 µm and images are analyzed by using Software called Leica TCS NT/SP SCANWARE (version 1.6.587). The stack of pictures' optical sectioning and 3D rendering were used to assess the localization of cyanobacterial strains that were present intracellularly. Adobe Photoshop CS3 version 10.0.1 was used to create and alter each (Fig 4).

Statistical analysis

The experiment was composed on wild species *Azolla caroliniana* twice with three triplicates. measurement was made during 0,5 10 15 and 20 day of life cycle with twice times, respectively. The software ANOVA was used to examine data from the assessment of antioxidant capacity, All the experiments was conducted in synchronized way in triplicates. Two-way analysis of variance (ANOVA) with significant difference $P \leq 0.005$ was used to compare the means of different treatment and control.

RESULTS AND DISCUSSION

Effect of Leds on growth rate

This work is the first investigation to find out the effect of monochromatic light on growth rate and doubling time of *Azolla caroliniana.* Culture is grown under different monochromatic light for 20th day of life cycle *and* their growth rate and doubling time was calculated. The results are presented in (Fig 2) where red LEDs treatment at (660-690) nm has shown least doubling time and highest growth rate.

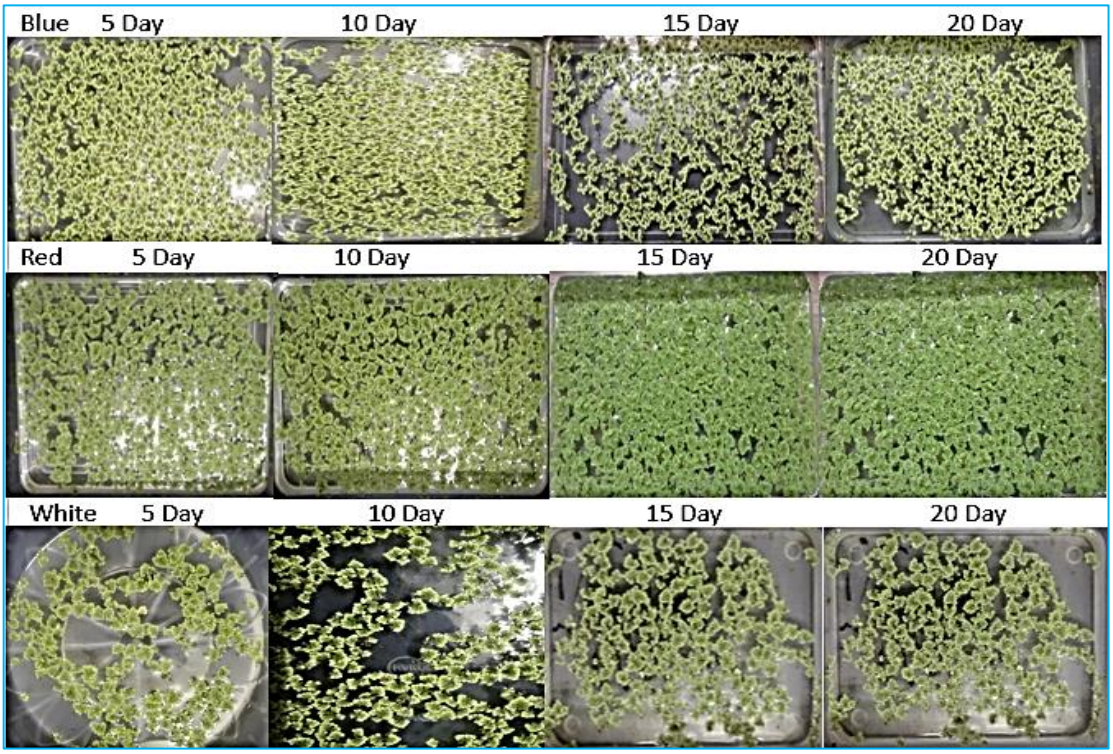

Fig 2 Effect of LEDs on growth rate and biomass of *Azolla caroliniana*

Effect of LEDS on DPPH and (APX) scavenging activity

The effect of monochromatic light on APx and DPPH radical scavenging activity of fern are measured under the experiment. The results show the concentration-dependent increase in DPPH and APx scavenging activity in methanol extract of *Azolla caroliniana* as shown in (Fig 3). Comparatively, to the other light treatments, red light increased the antioxidant characteristics APx and DPPH in plant *Azolla caroliniana*. The DPPH radical scavenging activity was enhanced under both blue and red light with significant difference from control ($P < 0.0001$). APx radical scavenging activity was also evaluated under current study, The highest antioxidant activity was found in leaf exposed to red light with significant difference ($P < 0.005$). No significant differences in the APx radical scavenging activity were observed among red and blue light-treated groups.

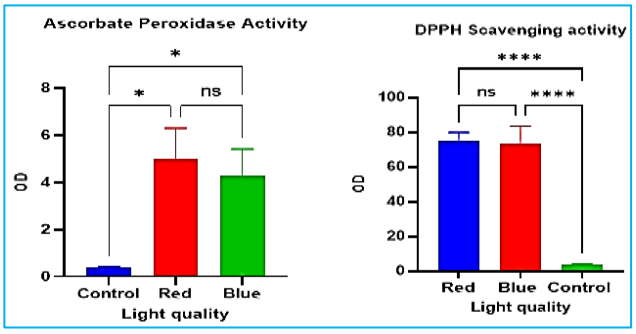

Fig 3 Antioxidant activity APx and DPPH for water: methanol analysis performed on *Azolla Caroliniana* treated with different red, blue, white light and control
*Letters denotes significant differences among different cultivators treated with LED light. Statistical significance difference was calculated by two-way ANOVA. P value was calculated with multiple variance test (α = 0.05). Values are the mean ± standard error from three replicates

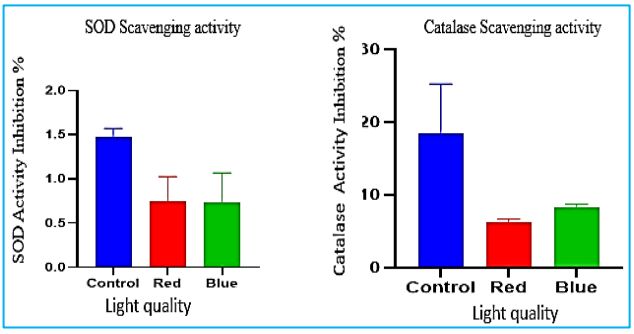

Fig 4 Antioxidant activity SOD and Catalase for water: methanol analysis performed on *Azolla Caroliniana* treated with different red, blue, white light and control. Statistical significance difference was calculated by two-way ANOVA. P value was calculated with multiple variance test (α = 0.05). Values are the mean ± standard error from three replicates

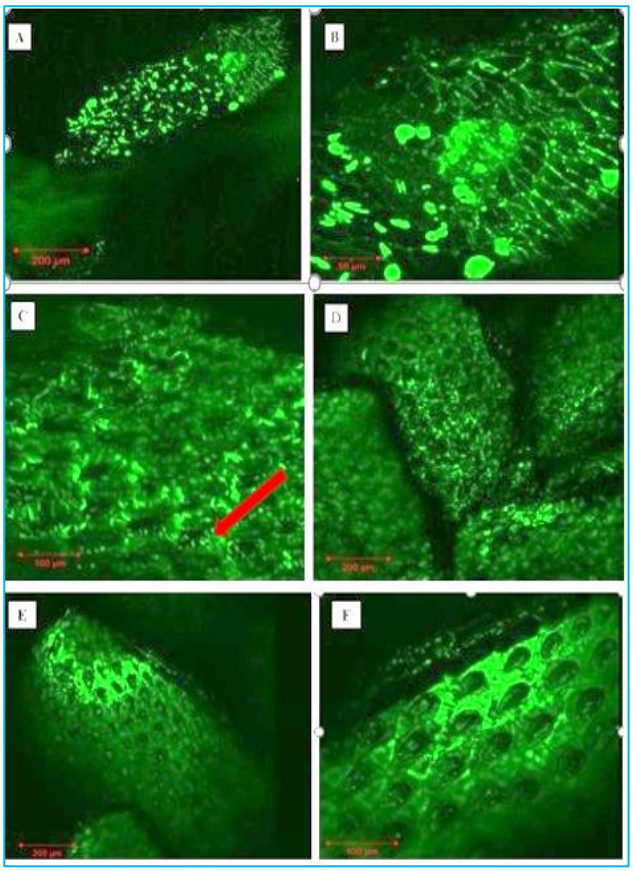

Fig 5 Confocal images analysis of leaf pore cavity and symbiosis of *Azolla* under of different monochromatic lights. (A-B) Control, (C-D) blue light, (E-F) red light

Effect of LEDS on SOD and catalase

Another important antioxidant activity studied in presented paper was to observe the effect of LEDs on SOD and Catalase activity of fern. Fig 4 shows that light treatment negatively influence the catalase and SOD activity in plant. The enzymatic activity of catalase (CAT) and SOD was least affected by the influence of different colors of light.

Effect of LEDS treatment on symbiosis and leaf pore of cavity of Azolla caroliniana

Symbiotic association with cyanobacteria is another important criterion of fern found in leaf cavity of *Azolla* was also observed in present research. This work is the first investigation to find out the effect of monochromatic light on symbiotic relationships and ultrastructure of fern. Results are summarized in (Fig 5). Highest auto fluorescence was observed in control plants, this native fluorescence is because of cyanobacteria which are residing within the leaf cavity of plant presented in (Fig 5 A-B). Whereas after the 20 days of LED exposure with red and blue monochromatic light fern show some drastic changes, the blue light exposure will cause the disappearance of cyanobacterium or reduction in their number and we obtained *Anabaena*- free plants presented in (Fig 5 C-D). In this study we visualize some ultrastructural cell wall projection within the pocket, schematic illustration in (Fig 5C). (Fig 5 E-F) represent the effect of red-light exposure which results in fully formed leaf pocket with some internal projection.

Light play important role in development and growth of plant, while among all the light the red and blue light are extensively studied by researcher [19], [24], [36]. Monochromatic light (red, blue and white) has significant impact on growth rate and doubling time of *Azolla caroliniana*. Our study concluded that (660–690) nm red LEDs shows the maximum impact on doubling time, comparatively with blue and white LED., showing that red light may favorably affect plant development in terms of growth [20], [28]. Moreover, proper light intensity, color, light source, and regimen have possibly improved the antioxidants, medicinal property and prevention of ROS-related diseases. The Light elicitation plays determining role in antioxidant production in plants [13], [21]. Therefore, the aim of the present study was to identify the effect of different monochromatic light regimen (white, blue, and red)

on antioxidant capacity of *Azolla caroliniana.* Increased in plant photobiology research revealed that various plant photoreceptors are studied well in respond to light, blue light response to phototropin or cryptochrome and red light for phytochromes [8].

Both red and blue light drastically improve APx and DPPH radical scavenging activity in plant and therefore applied as therapeutic application, Similar results were obtained by [23] when rice was treated with high concentration of Nacl will show over expression of cytosolic APx activity. Similarly, cAPX gene was strongly activated in leaves of sweet potato under high temperature stress and lead to increase in ascorbate activity [1], [26], [37] reported that red led enhances the DPPH activity in lettuce compare with blue light, similarly Achillea show highest DPPH activity under different abiotic stress condition reported by [17]. *Ocimum basilicum* DPPH free radical scavenging activity was greater under red light than under blue light. Alternatively [30] reported in *R. hongnoensis* DPPH free radical scavenging activity was increased under blue light.

Azolla leaf cavities contain symbiotic associations with cyanobacterial, this association is permanent and exists throughout the entire life cycle of the pteridophyte, regardless of whether sexual reproduction or vegetative fragmentation [27]. Based on the confocal microscopic investigation microscopy in this study we visualize some ultrastructural cell wall projection within the pocket, same projection reported in *Azolla* species by [35] under the salt stress condition but do not produce under normal conditions. [35] reported that projections are composed of pectin protein found on the cell walls of the specialized teat cells bordering the pore and have secretory role, indicative of a high metabolic activity and mechanically seal the cavity to prevent symbiotic evacuation and access the entry from outside [35]. Previous study also reported the presence of morphologically identical projections appear under stress condition or pathogen infection by in wounding, grafting, ozone exposure and fungal infection. Although several theories have been put out, it is still unclear how these projections are formed and might be play role in defense mechanism [22].

In our study we observed a significant change of autofluorescence of the cyanobacterium with effect of light treatment red light increase well-defined pore cavity in contrast to blue light which causes more conformational changes in pore cavity, to corroborate our observations based on cyanobacterial autofluorescence, we quantified the blue and red light will affect the symbiotic association of cyanobacteria. However, based on the data at hand, we cannot provide any conclusions on whether light may actively regulate the symbiotic mechanism and formation of teat cell or projection which has been previously suggested by [35], we suggest some more studies to observe the stress effect on association and its pore cavity.

CONCLUSION

This study aims on focusing of different LED light treatment on *Azolla caroliniana* growth, antioxidant and symbiotic association. Results obtained in this study suggest that red light induces maximum effect on growth and antioxidant properties of plant and which may be utilized in therapeutical application. Symbiotic relationships of fern were also regulated by light color and quantity. Light treatment induces the formation of ultrastructural cell wall projection within the pocket, suggested that under the stress condition these projections possibly play important role in defense mechanism for plants. Altogether, our observation concluded that red light illumination in fern is considered as beneficial for improving its medicinal property and more ecologically sustainable. However, more research based on photobiology by modifying the radiation would surely generate more cost-effective goals in all over development of fern.

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