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ORIGINAL PAPER

DTAF: an efficient probe to study cyanobacterial-plant interaction using confocal laser scanning microscopy (CLSM)

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Abstract A variety of microscopic techniques have been utilized to study cyanobacterial associations with plant roots, but confocal laser scanning microscopy (CLSM) is the least used due to the unavailability of a suitable fluorescent dye. Commonly used lectins have problems with their binding ability with root cells and their visualization under CLSM. DTAF (5-(4,6-dichlorotriazinyl) aminofluorescein) is a fluorescent dye that has been widely used for staining various biological samples for fluorescent microscopy. It reacts with polysaccharides and peptides at ordinary conditions. The possible application and efficiency of DTAF for CLSM studies were examined in various aspects of cyanobacterial-plant interactions. Seedlings of *Pisum sativum, Vigna rediata* and *Triticum aestivum* were co-cultivated and stained with DTAF as a fluorochrome. Extracellular and intracellular interactions of cyanobacteria and the plant root surface were observed by CLSM. Results were compared with staining by other commonly used lectins. Advantages of the use of DTAF over other stains are its penetration into root tissues and binding with polysaccharides, mainly the cellulose. The staining was smooth, which clearly showed minute details on the cell of surface and root hairs with higher resolution. The emission wavelength for DTAF is 517 nm, which is highly advantageous as cyanobacteria have auto-fluorescence at 665 nm, and both can be simultaneously used in CLSM by visualizing in different channels. This worked efficiently with all three plants used and with filamentous and unicellular cyanobacterial strains. Cyanobacterial presence was not only clearly observed on the root surface, but also inside the root tissue and epidermal cells. The easy protocol and absence of tissue processing make DTAF a useful probe for studies of cyanobacterial associations with plant roots by CLSM.

Keywords CLSM · Cyanobacteria · Cyanobacteria-plant associations · DTAF · 5-(4,6-dichlorotriazinyl) aminofluorescein · Fluorescent dye fluorescence

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Introduction

Cyanobacteria are commonly present in almost every environment, and they are reported to form associations with members of all kingdoms of life [18]. Their associations with roots of different plants are important but have not been very well studied. Different techniques have been employed to study the nature of cyanobacterial associations. Among the most fascinating of these is the direct observation of these associations by high resolution microscopy. As the cyanobacteria are bluish green in color, their presence on the roots can be seen under light microscopy. Electron microscopy is a very powerful tool in this regard, and both SEM (scanning electron microscopy) and TEM (transmission electron microscopy) have been equally utilized. SEM has been used to observe the external associations of cyanobacteria, which clearly demonstrated the fine details of the associations [6, 16]. TEM has been

used to locate the cyanobacterial presence inside the host tissue [11]. Although these techniques can give better visualization of cyanobacterial associations and especially their presence on the surface, they need a lot of time and effort for sample preparation and careful sectioning for intracellular localization of cyanobacteria [9]. Confocal laser scanning microscopy (CLSM) is another powerful tool not only for 3D localization of fluorescent organisms, but also for items dyed with fluorescent labels [4]. This technique allows in situ observation of the sample surface and in the depth of the sample with minimal sample preparation and without any artifacts. It also offers the possibility of non-invasive optical sectioning by subtracting out-of-focus planes of the image [9, 14]. By exploiting natural pigment fluorescence emitted by cyanobacteria (chlorophyll-*a* and phycobilins) with an emission wavelength of 665 nm, CLSM can be used for their detection in any sample [17]. In spite of the potential of this technique to study cyanobacteria, there are some limitations to its application to the study of plant-microbe interactions. The main hindrance is the availability of a suitable fluorescent probe, which on the one hand can bind specifically with the plant cells and on the other hand shows excitation wave lengths different from that of cyanobacterial auto-fluorescence so that both can be visualized simultaneously. DTAF (5-(4,6-dichlorotriazinyl) aminofluorescein) is a fluorescent dye that has been used for staining various biological samples for not only fluorescent microscopy, but also for qualitative and quantitative measurement of microbes and their fractions [3]. It mainly reacts with polysaccharides and peptides at ordinary conditions [2, 15]. This dye has an excitation wavelength of 493 nm and emission wavelength of 517 nm, which is quite different from the 665-nm emission wavelength of cyanobacterial autofluorescence, which led us to hypothesize that it may act as a fluorochrome in CLSM [8]. The objectives of the current research were to investigate the possibility of using DTAF as a fluorochrome dye in CLSM for imaging cyanobacterial associations with plant roots and to observe the intracellular invasions by these cyanobacteria.

Materials and methods

Cyanobacterial strains and plants used

Cyanobacterial strains isolated in our laboratory were used in this study, and these include filamentous strains, i.e., *Leptolyngbya* sp. MMG-1 (Genebank accession no. FJ839352) and *Phormidium* sp. MMG-4 (Genebank accession no. FJ839355) and unicellular strains *Chroococcidiopsis* sp. MMG-5 (Genebank accession no. FJ839356) and *Synechocystis* sp. MMG-8 (Genebank accession no. FJ839359) [1]. Certified seeds of wheat (*Triticum aestivum* var Uqab-2000), pea (*Pisum sativum* var. Climax) and mung bean (*Vigna radiata* var NM-92) were obtained from the Punjab Seed Corporation, Lahore, Pakistan.

Co-cultivation of cyanobacteria and seedlings

Seeds of all three plants without any sign of physical or pest damage were selected as healthy seeds, and their surface was sterilized by washing them for 5 min in 0.1% $HgCl_2$ followed by repeated washing with sterile distilled water. Sterile seeds were transferred to Petri dishes containing sterile Whatman filter paper no. 1. Plates were kept in the dark to let the seeds germinate. After germination, the plates were transferred to the fluorescent light 16:8 h light:dark period and light intensity of 200 μE m^{-2} s^{-1} at 25°C. Ten-day-old seedling roots were suspended in 50-ml tubes containing 25 ml of tenfold diluted BG11 (N^+) medium. Eighteen-day-incubated cyanobacterial cultures were harvested at 10,000 g for 10 min and resuspended in 10 ml of sterile MQ grade water. Cyanobacterial strains were added to the suspended seedling roots to a final concentration of 2 μg chlorophyll-*a* (chl-*a*) ml^{-1}. Co-incubation of the seedling roots and the cyanobacteria was carried out at 25°C at a 16:8 h light-dark cycle (200 μE m^{-2} s^{-1} light intensity). After 7 days of incubation the seedlings were harvested, and the roots were excised. Loosely attached cyanobacteria were removed by washing with sterile MQ grade water by squeeze bottle, and the roots were subsequently used for assessing colonization.

Sample preparation

Excised roots were cut in pieces of equal length (5 mm) and washed once with phosphate buffer. To stain the root cells, excised roots were stained with fluorescent dye DTAF [5-(4, 6-dichlorotriazinyl) aminofluores] (catalog no. D-16, Invitrogen Corporations, Carlsbad, CA) by keeping samples in 1 mM DTAF solution in carbonate buffer, pH 9 overnight. Excess stain from the sample was removed by washing with carbonate buffer, pH 9 twice. For comparison, staining was done with other commercially available fluorescent stains and commonly used fluorescently labeled lectins. Excised roots were stained with fluorescent dye FITC conjugated-*Canavalia ensiformis* lectin (ConA-FITC) by keeping samples in 1 mM ConA (catalog no. F-1104-25, EY Laboratories Inc., USA) solution in phosphate buffered saline, pH 8 for 20 min. Excess stain from the sample was removed by washing with phosphate buffered saline twice. For staining with TRITC conjugated-*Cytisus*

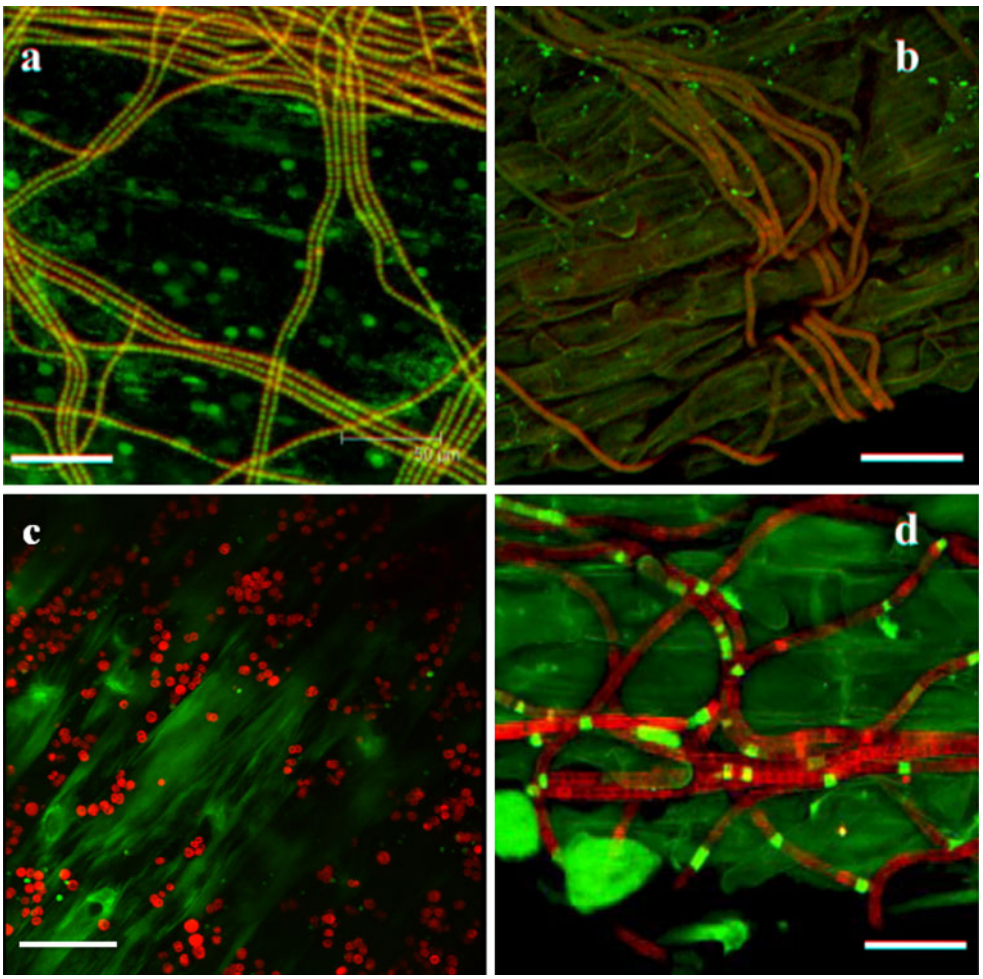

Fig. 1 Comparison of different dyes used in staining *Phormidium* sp. MMG-4 filaments on wheat roots. **a** Staining with SYTOX Green (*scale bar* 50 μm), **b** staining with ConA-FITC (*scale bar* 50 μm), **c** staining with CSA-TRITC (*scale bar* 50 μm), and **d** staining with DTAF (*scale bar* 50 μm)

sscoparius lectin (CSA-TRITC), excised roots were kept in 1 mM CSA (catalog no. R-3201-1, EY Laboratories Inc, USA) solution in phosphate buffer, pH 8 for 20 min. Excess stain from the sample was removed by washing with phosphate buffer twice. Staining the root samples with SYTOX® Green was accomplished by keeping samples in SYTOX green (catalog no. S-7020, Invitrogen Corp., USA) solution in Tris buffer for 20 min. Excess stain from the sample was removed by washing with Tris buffer twice.

Confocal laser scanning microscopy and image analysis

CLSM was performed using a TCS-NT microscope (Leica, Heidelberg, Germany) equipped with an argon-krypton laser. For simultaneous imaging of emission fluorescence from fluorescent staining and autofluorescence of cyanobacterial chlorophyll-*a* and phycobiliproteins, root sections were excited by beams of wavelength 488 nm. Emitted wavelengths from fluorescent dyes were collected using band-pass filter 530/30 and cyanobacterial natural fluorescence with a 590-nm-long pass filter. Images were obtained from the same field at different depths. Cyanobacterial extracellular colonization on the root surface was observed, and stacks of images were generated up to a depth of 5–10 μm, whereas for imaging the intracellular colonization, the stack of images was obtained up to a depth of 40–50 μm. The acquired images were analyzed with Leica TCS NT/SP SCANWARE (version 1.6.587) software. Overlaid images were generated by the outputs of two channels. Maximum projection algorithm, which determines the maximum of all intensity values in a stack of sections and displays them in one single image, was applied. Localization of cyanobacterial strains present intracellularly was analyzed by 3D rendering of the stack of images and their optical sectioning. All figures were produced and edited with Adobe Photoshop CS3 version 10.0.1 [1].

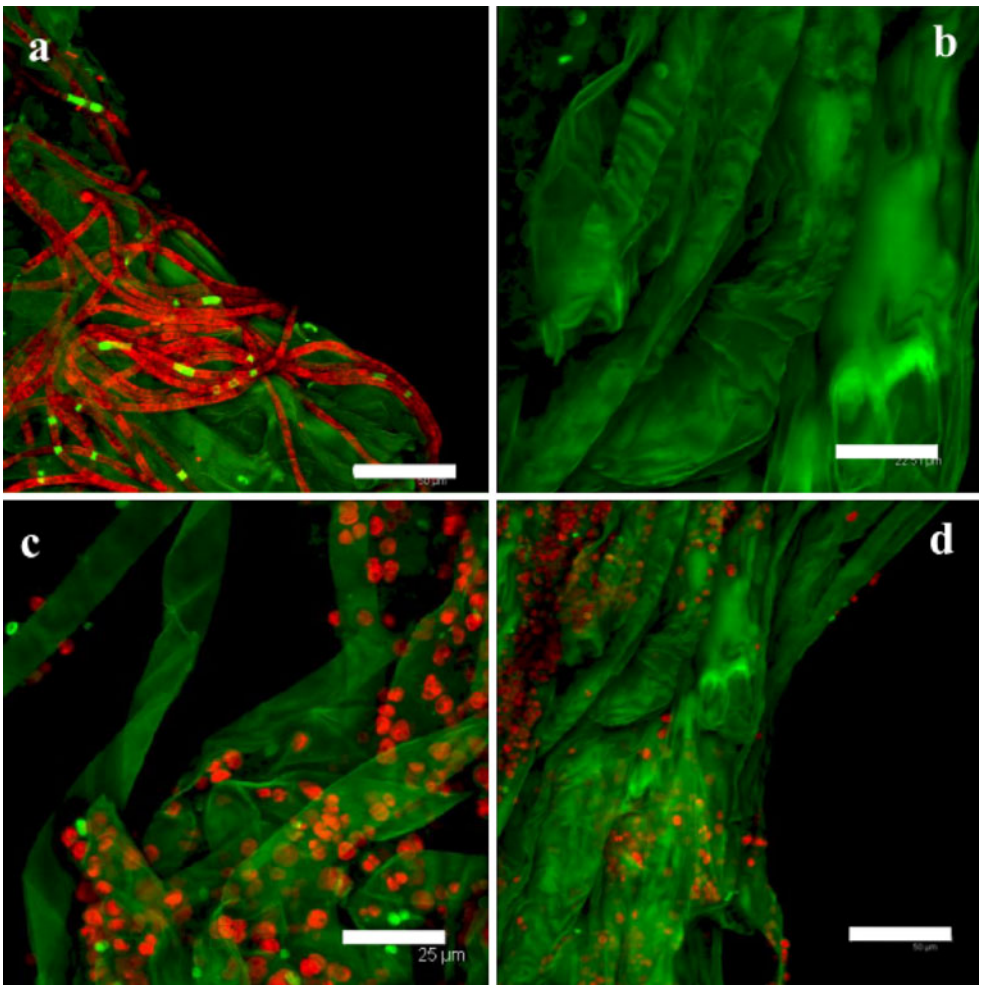

Fig. 2 Staining results with DTAF. **a** Presence of *Phormidium* sp. MMG-4 on mung bean roots (*scale bar* 50 µm), **b** staining of pea root surface and cells (*scale bar* 22.5 µm), **c** visualization of mung bean root hairs along with *Synechocystis* sp. MMG-8 cells on pea root hairs (*scale bar* 25 µm) and **d** presence of *Chrococcidiopsis* sp. MMG-5 cells on mung bean (*scale bar* 50 µm)

Results

Cyanobacterial associations with all three plants roots were observed by CLSM. The resolution and details visible after staining with DTAF were significantly better than staining by other lectins and fluorescent dye. The DNA binding dye SYTOX Green could only stain the DNA, i.e., the chromatin material, in case of cyanobacteria and nucleus in case of plant root cells. This staining resulted in a superficial overview of the cyanobacterial associations with plant roots as shown in Fig. 1a, the filamentous strain *Phormidium* sp. MMG-4 attached to the wheat rhizoplane. Furthermore, it was very hard to differentiate their internal colonization in all three plants. Although the FITC conjugated-*Canavalia ensiformis* lectin (ConA-FITC) stained the root cells, the imaging was not of very high resolution. In this case there was background noise that reduced the clarity of the images. The main problem with this dye was that it not only stained the root cells, but also the cyanobacterial filaments, which resulted in poor resolution of cyanobacterial strains on the root surface. In this case only big ridges on the wheat root surface were differentiated with less prominent *Phormidium* sp. MMG-4 filaments (Fig. 1b). When the same kind of sample was stained with TRITC conjugated-*Cytisus scoparius* lectin (CSA-TRITC), it was found to be a much better stain compared to the previous ones. This stain showed much less affinity with the cyanobacterial strains (*Chroococcidiopsis* sp. MMG-5), and plant cells were visible with significantly high brightness (Fig. 1c). This stain worked equally with all three tested plants. Imaging results after staining with DTAF were remarkable when compared with staining results by the lectins (Fig. 1d and 2a). The root cells were sharply stained with very fine structural details clearly visible in case of all three plant roots (Fig. 2a–d). Cyanobacterial cells were not stained, and they were also clearly visible

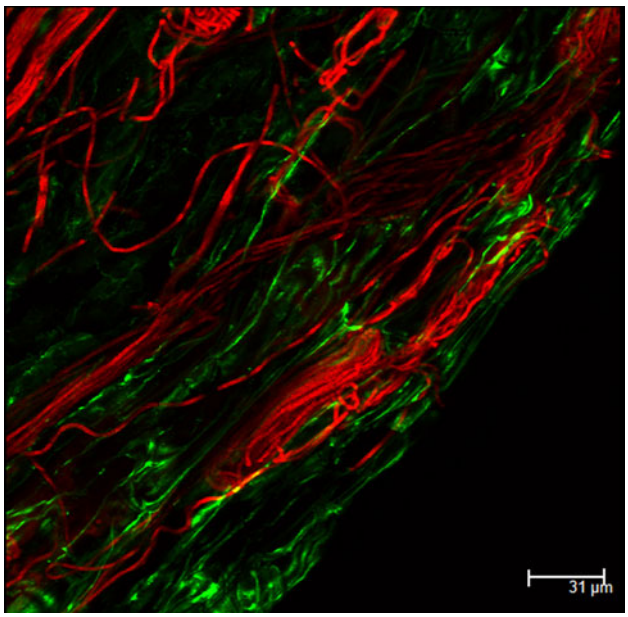

Fig. 3 Three-dimensional localization of filamentous cyanobacterial strain *Leptolygbya* sp. MMG-1 filaments inside the mung bean root cells (*scale bar* 31 µm)

with high resolution. This strain showed much less affinity with the cyanobacterial sheaths as there were only a few bright staining spots visible on the cyanobacterial filaments. The efficiency of this stain was further proved by imaging of the root hairs, which were visualized with very high resolution and with negligible background noise (Fig. 2c). Colonization of both unicellular strains, i.e., *Synechocystis* sp. MMG-8 and *Chroococcidiopsis* sp. MMG-5, was clearly observed on the root hairs of pea and on the rhizoplane of mung bean, respectively. An external as well as internal association of cyanobacteria with plant roots was also imaged with great detail. *Leptolygbya* sp. MMG-1 was visualized very clearly intracellularly in the mung bean root cells (Fig. 3).

Discussion

Images generated by the CLSM proved the effectiveness of this technique in observing the details of cyanobacterial associations with plant roots. Detection of the only fluorescently labeled parts of the sample removed the possibility of visualization of unwanted parts in the sample [9]. Complete absence of any natural fluorescence in the roots enables the spotting of fluorescent cyanobacteria in the sample. Furthermore, any stain that specifically binds to the plant cells and their parts and having an emission spectrum other than that of chlorophyll-*a* allowed the simultaneous imaging of both cyanobacteria and plant roots in separate channels of CLSM. Mostly lectins have been employed for fluorescent staining and observation of biological samples by CLSM. Lectins are exploited because of their binding ability to specific carbohydrates and proteins [10]. However, SYTOX green, a DNA stain, can be used along with the cyanobacterial auto-fluorescence as both have different emission spectra, but this only gives an idea about the extracellular presence of cyanobacteria. Among both of the lectins tested (FITC-ConA and TRITC-CSA), both stained the plant cells to a certain level and helped in visualizing the topology of the root surface, but CSA was found to be relatively better. FITC-labeled ConA lectin binds with mannose residues, which is an important component of the hemicellulose of plant cells [7]. It also stained the cyanobacterial sheath along with plant cells as the mannose is also an important residue in the majority of the cyanobacterial sheaths [12]. So after staining with ConA, the resolution of visualization of cyanobacteria-plant roots association was not very sharp, thus reducing its efficiency as a differential stain. Although TRITC-labeled CSA, a lectin that binds with N-acetylgalactosamine residue, stained the plant cells much better than ConA lectin, the cyanobacterial sheaths were also relatively less stained by this dye. The main drawback with both of these lectins was their inability to penetrate into the root cells, thus making these useless for intracellular localization of cyanobacterial strains. DTAF proved to be an excellent staining dye with many benefits over the lectins used. Advantages of the use of DTAF over other stains were its penetration into root tissues and binding with polysaccharides, mainly the cellulose. The staining was smooth, which clearly showed minute details on the cell of rhizoplane and root hairs with higher resolution. The emission wavelength for DTAF is 517 nm, which is highly advantageous as cyanobacteria have an auto-fluorescence at 665 nm and both can be simultaneously used in CLSM by visualizing in different channels. It worked efficiently with all three plants used and with filamentous and unicellular cyanobacterial strains. Cyanobacterial presence was not only clearly observed on the root surface, but also inside the root tissue and epidermal cells. The anionic fluorescent stain DTAF gave much less background staining and images with good contrast. DTAF has been reported to react directly with polysaccharides and peptides, and bind covalently to neutral amino groups of proteins especially on cell surfaces at ambient temperatures at pH above 9. The binding mechanism of DTAF described by Schumann and Rentsch [13] involves its covalent bond formation to primary hydroxyl functions of carbohydrates and N-termini of proteins. The covalent binding properties and the large number of binding places (hydroxyl functions, amino groups) particularly in carbohydrates make DTAF favorable as a robust (not removable by washing procedures), intensive fluorochrome for cells and other biological

Table 1 Comparison of different fluorescent dyes used and their efficiency in staining

Efficiency parameter		Stain			
		DTAF	ConA-FITC	CSA-TRITC	SYTOX Green
Specificity	Plant cells	High	Medium	High	High
	Cyanobacteria	Very low	Medium	Medium	High
Resolution of cyanobacterial cells	Unicellular cells (MMG-5, MMG-8)	High	Low	Low	Low
	Filamentous cells (MMG-1, MMG-4)	High	Low	Medium	Low
Resolution of plant cells	*Triticum aestivum*	High	Low	Medium	No
	Pisum sativum	High	Low	Medium	No
	Vigna radiata	High	Low	Medium	No
Penetration in plant cells	*Triticum aestivum*	High	Low	Low	High
	Pisum sativum	High	Low	Low	High
	Vigna radiata	High	Low	Low	High

samples [15]. Filamentous strain *Phormidium* sp. MMG-4 was clearly observed on the root surface, whereas *Leptolyngbya* sp. MMG-1 was observed clearly inside the cells. Minute details and folding of the root cell walls were distinctly visible on the roots of mung bean. Both unicellular strains were also imaged not only on the root cell surface, but also inside the cells. *Chroococcidiopsis* sp. MMG-5 cells were observed attached to the tiny root hairs of pea roots. The images produced by this stain (DTAF) were significantly better in terms of resolution and celerity compared to other lectins. DTAF has been reported to have a greater purity and stability than FITC-labeled dyes [5]. Putt [13] reported some problems in staining algal samples with DTAF, which was later found to be due to the staining conditions of the sample as optimal staining conditions were higher pH (>9) and longer staining time (>12 h) [15]. We found the best results for clear imaging of plant cells by staining the samples overnight at 9 pH.

Comparison of all fluorescent stains used showed differences in their efficiency, and DTAF proved to be best in different aspects (Table 1). It was observed that DTAF was found to be the most specific for plant cells and stained plant cells with high resolution, whereas other stains also bind to the cyanobacterial cells also to various extents. DTAF and all other stains worked equally with both unicellular and filamentous cyanobacterial strains, and their staining pattern was similar with all three plants used. The enhanced penetration power of DTAF also enables it to visualize the intracellular presence of cyanobacteria in plant root cells.

Conclusion

This study explored the potential of the fluorescent stain DTAF and its efficiency compared to other frequently used fluorescently labeled lectins for observing cyanobacteria-plant associations. It was found that this stain along with auto-fluorescence produced by the cyanobacterial pigments generated very fascinating images that can help study the nature of the relationship and fine details of such associations. With very simple sample preparation and an easy staining process, this dye can save both time and effort.

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