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Short Communication

Identification of Cyanobacteria Inhabiting Paddy Fields in Intermediate Zone and Dry Zone of Sri Lanka

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ABSTRACT: *Cyanobacteria have the ability to fix atmospheric nitrogen to plant available forms; thus, useful in producing bio-fertilizers especially for rice cultivation. In this study, cyanobacteria were isolated from soil samples collected from 23 paddy fields in Kurunegala, Matale, Anuradhapura and Polonnaruwa Districts under IL1, IM3, DL1b and DL1c agro ecological regions, respectively. Soil pH, EC, active C and exchangeable K contents varied considerably among soils from four regions. Culturable cyanobacteria were isolated using Blue Green medium (pH 7) and thirteen isolates were tentatively identified based on morphological characteristics. Oscillatoria was the most common cyanobacteria among sampling sites in IL1, IM3 and DL1c agro ecological regions. Microsystis was the most prevalent unicellular group in IL1 and DL1c agro ecological regions. In DL1b, unicellular Aphanocapsa was commonly found followed by filamentous Pseudanabaena and Oscillatoria. From this research thirteen axenic cultures were established for further studies. The diversity of cyanobacteria was high in the regions with high diversity of paddy cultivated environments.*

Keywords: *Paddy, biofertilizer, Cyanobacteria, N_2 fixation*

INTRODUCTION

Cyanobacteria thrive in favourable growth conditions in fresh water and wetland ecosystems including lowland paddy fields, which are manmade wetland ecosystems. They live as free-living organisms (e.g. *Aphanocapsa, Gloeocapsa* and *Merismopedia*) and in symbiotic associations (e.g. *Anabaena* sp. with *Azolla*) (Kulasooriya, 2011). Cyanobacteria are a morphologically diverse group. They can be grouped into uni-cells (e.g. *Synechocystis*), colonies of individual cells (e.g. *Aphanothece*), unbranched filaments (e.g. *Lyngbya*), aggregations of multiple filaments in a common sheath (e.g. *Microcoleus*), false-branched forms (e.g. *Scytonema*) and true branched forms (e.g. *Stygonema*), and those forming baeocytes (endospores) (e.g. *Myxosarcina*) or forming exospores (e.g. *Chamaesiphon*) (Wehr and Sheath, 2002). Cyanobacteria are major nitrogen (N_2) fixing prokaryotic organisms in the paddy field water and the uppermost soil layer (Roger, 1996). Their diversity in the rice paddies and nitrogen supplying potentials vary according to the growth stage of the rice plant

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and physico-chemical environment of the paddy soil (Prasanna *et al.,* 2009; Song *et al.,* 2005).

Cyanobacteria have been used in formulations of biofertilizers especially for rice cultivation (Kulasooriya and Magana-Arachchi, 2016). In addition to N_2 fixation, cyanobacteria stabilize soil surface and increase water holding capacity. Some cyanobacteria excrete plant growth promoting substances such as growth hormones, vitamins, amino acids, and organic acids, and suppress weed growth, increase available phosphorous level in soil and decrease the effects from soil salinity (Saadatnia and Riahi, 2009). Soil pore structure is improved by filamentous growth and secretion of adhesive substances. Endosymbiosis of some cyanobacteria with aquatic flora (e.g. *Azolla*), (Kneip *et al.,* 2008) found to be more successful in fixing nitrogen compared to free-living cyanobacteria. *Azolla* are widely used in China, certain parts in Philippines and Vietnam as a fertilizer (Kulasooriya, 2011).

Less colonization of the introduced environment during field application is a limiting factor for developing and popularizing biofertilizers or inoculants based on cyanobacteria (Naveed *et al.,* 2015). This can be due to failed acclimatization to prevailing geochemistry or competition and predation by native soil micro flora and micro-fauna (Kulasooriya and Magana-Arachchi, 2016). Isolating cyanobacterial species from local paddy fields is an essential initial step in developing biofertilizers as these microorganisms are well adapted to prevailing soil environmental conditions. Thus, the aim of the current study was to develop a culture collection of cyanobacterial isolates from paddy fields at intermediate and dry zones of Sri Lanka. The descriptive results on the morphological identification of cyanobacterial isolates and their distribution among different locations are presented in this paper.

METHODOLOGY

Sampling and sampling sites

Soil samples were collected from 23 paddy fields in Kurunegala (n=7), Matale (n=4), Anuradhapura (n=5) and Polonnaruwa (n=5) under IL1, IM3, DL1b, and DL1c agro-ecological regions respectively (Figure 1). The soils in the four locations belong to Batalagoda series (Typic Endoaquents), Matale series (Typic Rhodudalfs), Hurathgama series (Typic Endoaqualfs) and Kuda *Oya* series (Typic Endoaqualfs), respectively (Mapa *et al.,* 2005 and 2010). Paddy fields under rain-fed and major and minor irrigation were used whenever available for sampling to obtain high diversity in culture collection. After geo-referencing the sampling site, 3 sub samples were collected from the surface soil layer (0-10 cm) to make a composite sample per field and immediately transported to the laboratory on ice and stored in 4 °C until analyses.

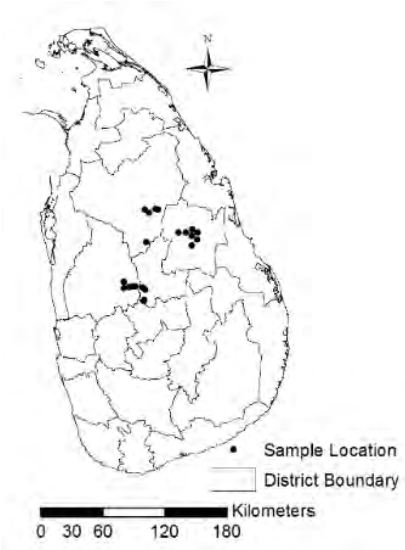

Figure 1. Distribution of sampling sites

Soil analyses

Each sample was analyzed for moisture factor (MF), electrical conductivity (EC) and soil pH (1:5 soil: water). Soil active C content was analysed by Permanganate Oxidizable C (POXC) technique (Culman *et al*., 2012) and exchangeable K of soil samples were measured using Ammonium acetate method (Lathiff, 2007a).

Sample preparation and isolation of cyanobacteria

Sub samples of soils (10 g) were thawed and mixed with 90 ml autoclaved deionised water and the soil suspension was allowed to settle for 10 minutes. Prepared soil suspension was filtered using Whatman No. 1 filter paper and the filter paper with filter cake was transferred to a flask with 200 ml of Blue Green medium with nitrogen (BG 11, pH 7.5) (Meeks, 2017). Liquid cultures were kept on shaker at 100 rpm and 2000 lx light conditions (16/8 h) at 28 °C. After two weeks of culturing a dilution series was prepared to determine the optimum dilution of the culture for inoculation on solid media (1% bacteriological agar) using spread plate method. Plates were incubated in replicates at 28 °C and illuminated with fluorescent light (2000 lx) (16/8 h).

Identification of culturable Cyanobacterial species by morphological observations

A light microscope fitted with a digital camera was used for the morphological identification, and images were captured at 40, 100 and 400× magnification. Identification of different species/genotypes of culturable cyanobacteria was performed based on the keys developed by Wehr and Sheath (2002). Morphologically identified cyanobacterial species were further purified by streaking on solid medium until an axenic culture was obtained. Axenic liquid cultures were prepared by transferring single colonies from axenic solid cultures to 5 ml of BG 11 liquid medium.

RESULTS AND DISCUSSION

Cyanobacteria are ubiquitous as they have a great adaptability to environmental variations. Paddy field ecosystem facilitates suitable environmental conditions including light, temperature, water and nutrient availability for the growth and the establishment of cyanobacteria (Kondo and Yasuda, 2003). Thus cyanobacteria are highly abundant in paddy soils. In soil ecosystem, cyanobacteria are important players in fixing nitrogen, stabilizing the soil surface, increasing water holding capacity and promoting plant growth via secretion of plant growth promoting substances. Factors including dry/wet season, soil pH, available P and N fertilizer input are found to affect the growth of cyanobacteria in paddy soils (Roger, 1996). Medium pH is a very important factor for the growth and establishment and the diversity of cyanobacteria. Most suitable pH has generally been reported to range from neutral to slightly alkaline for their growth (Kaushik, 1994). In culture media, the optimal pH ranges from 7.5 to 10 with a lower limit of 6.5 to 7.0.

In the present study, soil samples were collected from 23 locations and soil physico-chemical properties varied considerably among these locations (Table 1). Soil pH ranged from neutral to slightly acidic. Higher EC values were observed in paddy soils from Anuradhapura and Pollonnaruwa Districts than from Matale and Kurunegala. Observed values for soil pH and EC across the four districts were within the favourable range for paddy cultivation (less than 0.15 dS/m of EC, 6-7 of soil pH), while exchangeable K was slightly lower than the optimum range (80-160 mg K/kg) in sampling sites in Anuradhapura and Polonnaruwa (Lathiff, 2007b). These variations in soil parameters could be resulted due to edaphic factors, climatic factors and physiographic factors of crops. Also it could lead to harbour diverse groups of microorganisms.

Table 1. Moisture content, pH, EC, active C and exchangeable K (mean±standard deviation) of soils collected from paddy fields in Kurunegala, Matale, Anuradhapura and Polonnaruwa under IL1, IM3, DL1b and DL1c agro ecological regions, respectively.

District	Moisture Factor	pH	EC dS/m	Active C (ppm)	Exchangeable K (ppm)
IM3 (n=4)	1.64±0.19	6.68±0.26	0.012±0.002	568±283	90±20
IL1 (n=7)	1.55±0.15	6.83±0.18	0.018±0.013	771±322	97±32
DL1b (n=5)	1.52±0.19	6.82±0.54	0.075±0.043	1111±501	72±39
DL1c (n=7)	1.71±0.18	6.61±0.13	0.037±0.022	1479±319	64±32

A total of 80 isolates were made collectively from all locations and these were assigned to 13 genera tentatively based on morphology. The isolates included 6 unicellular (*Chroococcus, Aphanocapsa*, *Aphanothece*, *Synechococcus*, *Johannesbaptistia*, and *Microsystis*) (Figure 2) and 7 filamentous (*Lyngbya*, *Oscillatoria*, *Leptolyngbya*, *Pseudanabaena*, *Anabaena*, *Spirulina* and *Nostoc*) (Figure 3) cyanobacteria.

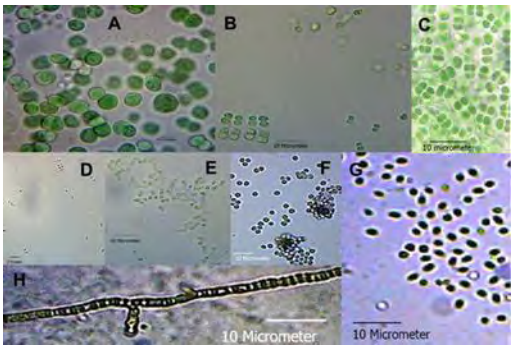

Figure 2. Unicellular cyanobacteria (A, B, and C) *Chroococcus*, (D) *Aphanothece*, (E) *Synechococcus,* (F) *Aphanocapsa*, (G) *Microsystis*, (H) *Johannesbaptistia*

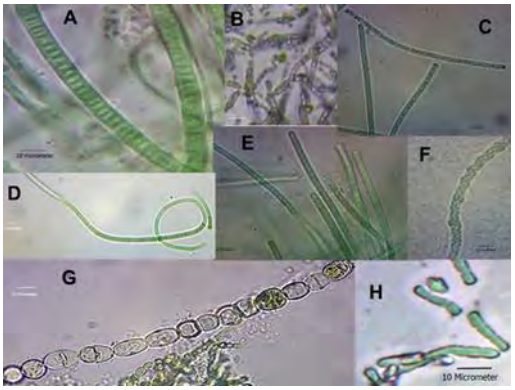

Figure 3. Filamentous cyanobacteria (A and E) *Oscillatoria*, (B) *Anabaena*, (C) *Leptolyngbya*, (D) *Lyngbya*, (F) *Spirulina,* (G) *Nostoc and* (H) *Pseudanabaena*

The prevalence of different types of cyanobacteria in the four regions is shown in Figure 4. Filamentous *Oscillatoria* was the most common cyanobacteria among sampling sites in IL1, IM3 and DL1c agro ecological regions; whereas, in DL1b region unicellular *Aphanocapsa* were more commonly found followed by filamentous *Pseudanabaena* and *Oscillatoria*. *Microcystis* was the most prevalent unicellular group in IL1 and DL1c regions. Several unicells i.e. *Synechococcus, Aphanothece* and *Johannes baptistia* and filamentous types i.e *Anabaena*, *Lyngbya* and *Spirulina* were found only in paddy fields from Kurunegala district under IL1 agro ecological region indicating high diversity of cyanobacteria. The occurrence of different genera based on morphological classification is in the order of Kurunegala > Anuradhapura > Polonnarua > Matale. This was expected because the diversity of paddy growing environments among sampling sites in Kurunegala was higher than other three regions. Sampling sites in Kurunegala represented rice cultivated under rain-fed, minor and major irrigation schemes which leads to different cropping systems (rice-rice, rice-other filed crop and rice-fallow). Whereas, sampling sites in Anuradhapura and Polonnaruwa were under major or minor irrigation schemes and those in Matale were rain-fed paddy fields.

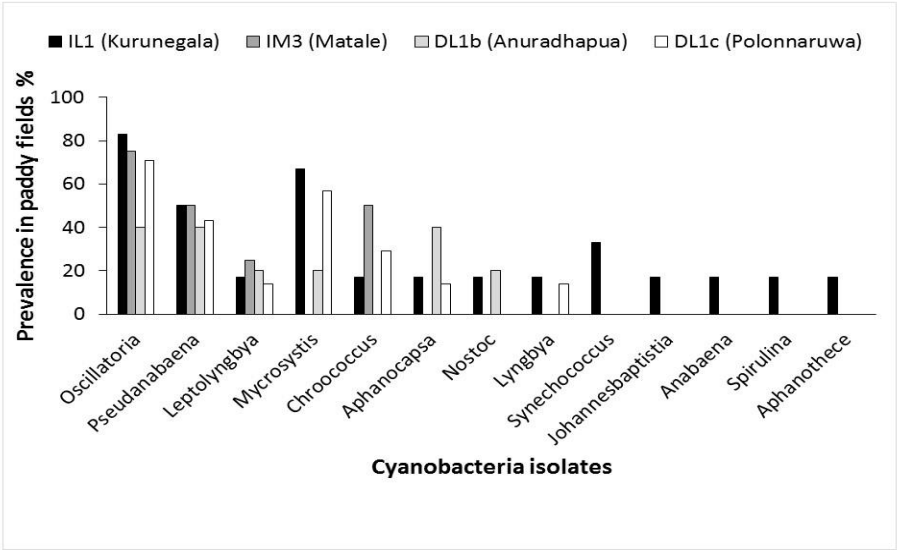

Figure 4. **Prevalence of different cyanobacteria among sampling sites fall under the IL1, IM3, DL1b and DL1c agro ecological regions in Kurunegala, Matale, Anuradharpura and Polonnaruwa Districts respectively.**

Cyanobacteria are found as a prominent group in rice soil microbial community (Kulasooriya and Maganarachchi, 2016). A recent study by Wanigatunge *et al.* (2014) has reported the cyanobacterial diversity in paddy fields and other wetland ecosystems including freshwater and brackish water in Sri Lanka using morphological and molecular identification. They found that Genus *Microcystis, Gleocapsa, Gloeothece, Leptolyngbya, Phormidium, Plectonema, Pseudanabaena, Anabaena* and *Calothrix* representing three cyanobacterial Orders namely, Chroococcales, Oscillatoriales and Nostocales, were prominent in paddy soils at *Gampola, Elpitiya,* and *Thotagamuwa* located in the Wet zone (Wanigatunge *et al.,* 2014). In the current study 13 tentatively identified cyanobacterial genera representing six cyanobacterial Orders (IL1, IM3, DL1b and DL1c) were found. The difference in prevalence could be partly due to the diversity of rice growing environments and related favourable growth conditions in Dry and Intermediate zones used in the present study. Further, the identification of cyanobacteria in the present study was based on morphology, which may seriously undermine the true diversity. A similar study by Prasanna *et al,* 2009 in India has reported that rhizosphere soil samples collected at different rice growing areas were dominated by culturable heterocystous forms belonging to *Anabaena* and *Nostoc,* while other heterocystous forms *Hapalosiphon*, *Westiellopsis* and *Calothrix* and non-heterocystous forms, *Oscillatoria* and *Phormidium* occurred in the rhizosphere depending on the geographical locations and rice varieties. In addition, cyanobacterial phylotypes found in surface (0–5 cm) and sub-surface soils (10–15 cm) in rice fields have shown a seasonal variation with the crop growth (Song *et al.,* 2005). They reported abundance of *Leptolyngbya* and *Nostoc* throughout the season and *Synechococcus, Phormidium* and *Synechosystis, Scytonema*, *Cyanothece*, *Microcoleus*, *Chamaesiphon*, *Spirulina*, and *Chroococcidiopsis* during different time periods using molecular methods. Thus the distribution and abundance of different cyanobacteria in paddy fields are found to be influenced by different eco-hydrological factors such as soil properties, crop growth, water regime, type of fertilizers

(Song *et al.,* 2005) which determines the spatio-temporal variations in total bacterial and fungal community composition in paddy soils (Balasooriya *et al.,* 2016).

In the studied rice fields, the most frequently found culturable cyanobacteria was *Oscillatoria* sp. *Oscillatoria* is inhabited by range of environmental factors including mild temperature (26 °C) (e.g. *Oscillatoria proboscides*), high temperature (38 °C) (e.g. *Oscillatoria corntiana*) and high dissolved oxygen level (e.g. *O. corntiana*) (Singh *et al.,* 2014). Average temperatures in intermediate zone (24–26 °C) and dry zone (28 °C) fall within the favourable range for *Oscillatoria*. In addition, aerated conditions might have prevailed in surface layer of rice fields due to channel of atmospheric air through aerenchyma in the leaves, stems and roots of rice plants and aerated irrigation water. However, shaking of culture flasks might have influenced the abundance of *Oscillatoria* as well. In previous studies, *Oscillatoria limosa* (Stal and Krumbein, 1981), *Oscillatoria* sp. (Kumazawa and Mitsui, 1985), *Oscillatoria* sp. strains UCSB8 and UCSB 25 (Gallon *et al*., 1991) were reported as aerobic nitrogen fixing cyanobacteria in mariner environments. Although *Oscillatoria* are frequently found in terrestrial sites including paddy fields, studies on their N_2 fixing potential are rare. Thus, evaluation of the isolated cyanobacteria for N_2 fixation ability is prospected. Furthermore, cyanobacteria are known as sources of plant growth promoting substances such as, auxin (e.g. *Anabaena* and *Nostoc*) (Ahmad and Winter, 1968), cytokinin (e.g. *Anabaena*) (Rodgers *et al.,* 1979) and gibberellins (e.g. *Anabaenopsis*) (Singh and Trehan, 1973). On the other hand, some cyanobacteria produce cyanotoxins, which include potent neurotoxins, hepatotoxins and cytotoxins (Bergman *et al.,* 1997). Thus, it is essential to identify the cyanobacterial isolates using molecular techniques and then screen them for potential beneficial (N_2 fixation, P solubilisation, growth hormone production, etc.) and harmful characters (e.g. cyanotoxin production) prior to utilizing them as biofertilizers.

CONCLUSIONS

Thirteen cyanobacteria genera were isolated and tentatively identified from paddy soil crust in the intermediate and dry zones of Sri Lanka based on their morphological characteristics. Among them, 6 cyanobacteria genera were unicellular (*Chroococcus, Aphanocapsa*, *Aphanothece*, *Synechococcus*, *Johannesbaptistia*, *Microsystis)* and 7 genera were filamentous types (*Lyngbya*, *Oscillatoria*, *Leptolyngbya*, *Pseudanabaena*, *Anabaena*, *Spirulina, Nostoc*). *Oscilatoria* was the most abundant type among sampling sites in IL1, IM3, DL1b and DL1c agro ecological regions falling under Kurunegala, Matale, Anuradhapura and Polonnaruwa Districts. The prevalence of different types of cyanobacteria is higher in paddy fields in the Intermediate zone compared to Dry zone. Hence, the diversity of cyanobacteria is high in the regions with high diversity of paddy cultivated environments. Molecular analysis for further identification of cyanobacteria isolates is prospected.

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