



Journal Homepage: -www.journalijar.com
**INTERNATIONAL JOURNAL OF
 ADVANCED RESEARCH (IJAR)**

Article DOI:10.21474/IJAR01/6255
 DOI URL: <http://dx.doi.org/10.21474/IJAR01/6255>



RESEARCH ARTICLE

IN-VITRO SCREENING OF ANTIFUNGAL ACTIVITY OF CYANOBACTERIAL EXTRACTS AGAINST SOME PHYTOPATHOGENIC FUNGI.

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Manuscript Info

Manuscript History

Received: 08 November 2017
 Final Accepted: 10 December 2017
 Published: January 2018

Key words:-

Antifungal, Cyanobacteria, Phyto-pathogens, Bio-fungicides, Bio-control agents.

Abstract

As a free living diazotrophic prokaryotic microorganism, Cyanobacteria produce a wide array of toxic chemicals and secondary or bioactive metabolites. Allelopathic interactions involving Cyanobacterial flora are being explored for their pharmaceutical and environmental significance. Cyanobacterial allelopathy can be regarded as one of the significant factors influencing their dominance in diverse habitats and as unique producers of a variety of allelochemicals that can be utilized as eco-friendly bio-control agents. In present work detrimental effects of locally isolated Cyanobacterial strains were evaluated against plant pathogenic three fungal isolates (*Fusarium oxysporum*, *Sclerotium rolfsii* and *Rhizoctonia solani*). It was observed that the crude extracts of four Cyanobacterial isolates (*Microcystis aeruginosa*, *Oscillatoria princeps*, *Anabaena anomala* and *Nostoc calcicola*), were capable of diminishing the growth and further development of phyto-pathogenic fungal isolates. Whereas *M. aeruginosa* showed more antifungal activity compared to other cyanobacterial strains and its Methanol extracts were observed more efficient against local fungal isolates *Fusarium oxysporum* (% MI – 88.46 ± 0.24) and *Sclerotium rolfsii* (83.42) as compared to *Rhizoctonia solani* (78.33). Toxic as well as allelopathic potentialities of cyanobacteria have needed to be further investigation that can offer promising solutions to bio-control against pathogenic microorganisms, providing novel bio-fungicides.

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Introduction:-

Agriculture in Chhattisgarh is an important economic sector, most of the population living on its revenues. The most common crop of this state is paddy, so called 'Dhan Ka Katora'. Consequently Fungi are the main responsible agents for losses in agriculture and horticulture that can infect any part of the plant [1]. The fight against these fungi is based on the use of chemical pesticides. However, chemical pesticides sprayed into the air or discharged into the soil can be harmful to the environment and to humans as well.

After Green Revolution enormous progress with modern irrigation and fertilizer application has been made. The concern of pesticide/fungicide use with respect to human health and environment has brought increasing interest in alternatives by avoiding negative effects on the environment. Cyanobacteria are known to produce intracellular and

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extracellular metabolites with diverse biological activities such as antialgal, antibacterial, antifungal and antiviral activity [2][3][4]. Secondary metabolites from cyanobacteria are associated with toxic, hormonal, antineoplastic and antimicrobial effects [5]. Recently algae are one of the chief biological agents that have been studied for the control of plant pathogenic fungi, particularly soil-borne disease [6]. Cyanobacteria and eukaryotic algae produce bio-active compounds that have antifungal and antibacterial activity [7] against plant pathogens. Allelopathic impact of Cyanobacteria on pathogenic fungi was also studied [8]. Secondary metabolites influence other organisms in the vicinity and are thought to be of phylogenetically important. Kulik (1995) stated a number of reasons, Cyanobacteria and algae are suitable candidates for exploitation as bio-control agents of plant pathogenic fungi. The potential for using Cyanobacteria is the bio-control of plant pathogenic bacteria and fungi. Tiwari & Sharma (2013) [9] has observed the antifungal activity of *Anabaena variabilis* against plant pathogens. Screening of Cyanobacteria for antibiotics has opened a new horizon for discovering new drugs. Exploring antifungal efficacy of Cyanobacteria to control plant pathogenic fungi can prove to be an excellent option as they are easy to grow with minimum nutrients, cost effective, no side effects and environment friendly. This work describes the antifungal effect of Cyanobacterial isolates against phytopathogenic fungi.

Materials and Methods:-

Isolation and identification of microbes:-

For the selection of the experimental materials different species of Cyanobacteria and Fungi were isolated applying proper media and methods. Identification of isolated microbes was completed by microscopic observation.

Cyanobacteria:-

Soil samples were collected from various agro-fields of Bilaspur division. The samples were brought to laboratory in plastic vials /zipped polythene bag and washed with distilled water to prevent potential contaminants. Isolation of Cyanobacterial strain was performed in specific nutrient media (Allen & Arnon, BG-110 and Chu-10) and was stored as suspension culture in growth medium under prescribed condition [10] [11]. Isolated strains were identified by microscopic observation with the help of Key standardized by Desikachary (1959) and Anand (1989) [12, 13].

Fungal strains:-

The fungal strains were isolated from agronomic soil samples. They were grown in the laboratory in suitable media such as Potato dextrose agar media (PDA) and Sabouraud's agar media and fungal isolates were identified on the basis of morphological characteristics by microscopic observation. Taxonomic guides [14] were used to identify the isolated fungal culture.

Extraction:-

Axenic culture of Cyanobacterial isolates were raised in growth cabinet under laboratory condition. At the stationary phase of growth (approximately 30 days), Cyanobacteria biomass were harvested by centrifugation and dried in a hot air oven at 50°C over night. The dried biomass - 2g/25ml (w/v) of Cyanobacteria were extracted with 75% methanol and ethanol (HPLC grade). The extracts were sonicated for 20 min using ultrasonic micro-tip probe of 400 watt and centrifuged at 5000 rpm for 10 min. Supernatant was retained and the pellet re-extracted as before three times. Combined supernatant was evaporated to dryness at 40°C using rotary evaporator. Dried extracts were stored in labeled sterile vials in a refrigerator till further use [15].

Toxicity assessment:-

For testing the antifungal activities of crude extract of Cyanobacteria against identified fungal isolates with the help of laboratory methods and Carbendazim and Mancozeb (as fungicides) were used as control. The following method had been employed to confer the toxicity of crude extract of different Cyanobacterial isolates on identified bacteria & fungi.

Percent mycelial inhibition:-

Petri plates with medium were aseptically inoculated at the centre with mycelia disc of 5mm diameter taken from 96 hrs old culture. Plates were then incubated at $26 \pm 1^\circ\text{C}$ in BOD incubator and mycelial growth pattern was observed. Radial diameter was recorded twice perpendicularly after 96 hrs incubation using a transparent millimeter ruler. Percentage inhibition of mycelia growth was calculated.

Results and Discussion:-

Through examination of samples total 16 species of Cyanobacteria were isolated, out of which nine heterocystous form and seven non-heterocystous forms were identified as nitrogen fixing and non nitrogen fixing strains respectively (Table -1, Plate -1; Fig. – I to XVI).

Table - 1: Morphologically dissimilar Cyanobacteria isolated during the survey.

Thallus	Non Nitrogen fixing	Nitrogen fixing
Unicellular	1. <i>Aphanocapsa elachista</i> 2. <i>Aphanothece saxicola</i>	NIL
Colonial	3. <i>Microcystis aeruginosa</i>	NIL
Filamentous	4. <i>Lyngbya birgei</i> 5. <i>Lyngbya shackletoni</i> 6. <i>Oscillatoria princeps</i> 7. <i>Oscillatoria cortiana</i>	8. <i>Anabaena oryzae</i> 9. <i>Anabaena anomala</i> 10. <i>Anabaena unisporea</i> 11. <i>Aulosira prolifica</i> 12. <i>Calothrix elenkinii</i> 13. <i>Cylindrospermum indicum</i> 14. <i>Gloeotrichia echinulata</i> 15. <i>Nostoc calcicola</i> 16. <i>Nostoc ellipsosporum</i>

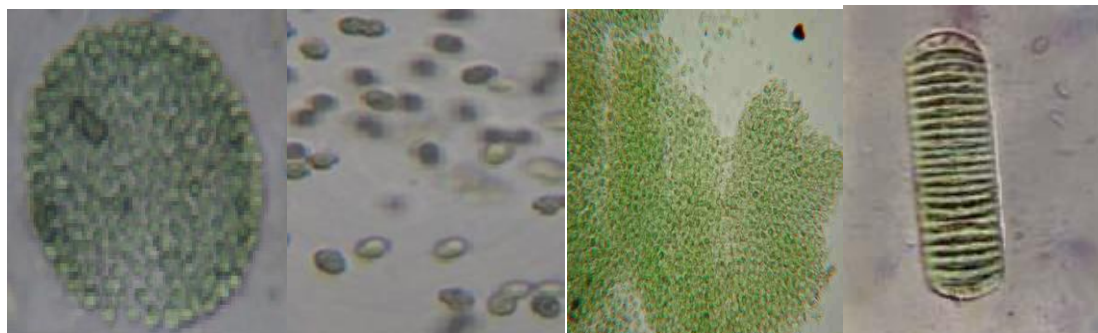


Fig.-I[*Aphaenocapsa elachista*]
Fig.-II[*Aphanothece saxicola*]

Fig.-III[*Microcystis aeruginosa*]
Fig.-IV[*Lyngbya birgei*]

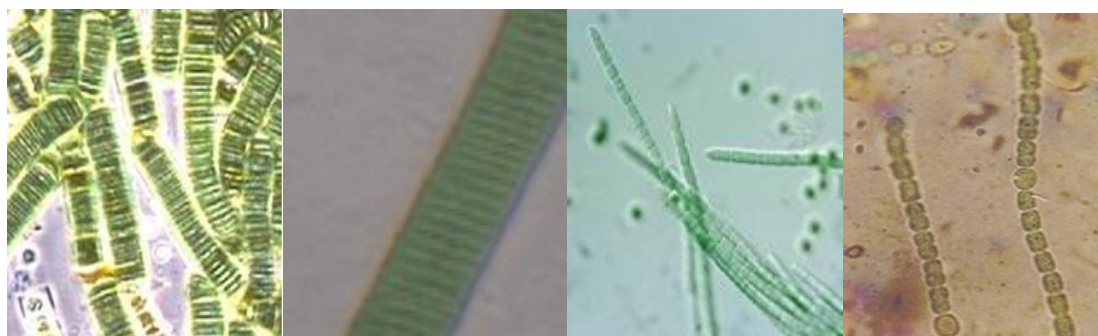


Fig.-V[*Lyngbya shackletoni*]
Fig.-VI[*Oscillatoria princeps*]
Fig.-VII[*Oscillatoria cortiana*]

Fig.-VIII[*Anabaena oryzae*]

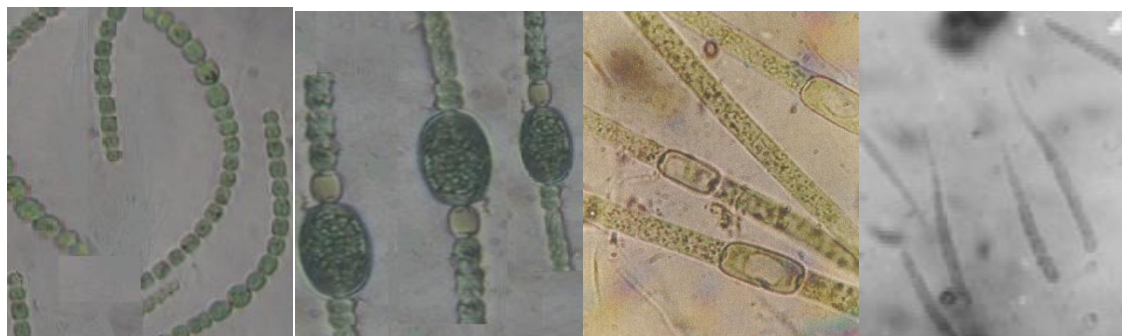


Fig.-IX [*Anabaena*]
Fig.-X [*Anabaena anomala*]
Fig.-XI [*unisporea*]
Fig.-XII [*prolififica*]

Fig.-XII [*Aulosira*]
Fig.-XIII [*Calothrix elenkinii*]

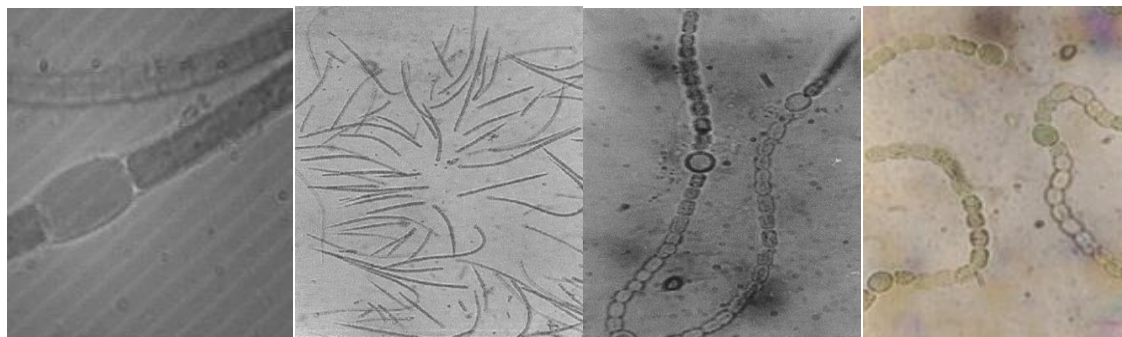


Fig.-XIII [*Cylindrospermum indicum*]
Fig.-XIV [*echinulata*]
Fig.-XV [*Gloeotrichia calcicola*]
Fig.-XVI [*Nostoc ellipsosporum*]

Plate - 1: Fig. - I – XVI

Simultaneously total 6 fungal species (*Aspergillus niger*, *Penicillium notatum*, *Mucor sp.*, *Sclerotium rolfii*, *Fusarium oxysporum* and *Rhizoctonia solani*) were isolated and identified on the basis of their morphological features (Table -2, Plate -2; Fig. – I to VI).

Table 2:-Characteristic features of fungal isolates.

Name Of Fungi	Taxonomic Status	Colour	Microscopic Observation
<i>Aspergillus niger</i>	Ascomycetes	dark to pale yellow in early stage while became black at maturity	Jet black conidia, reverse usually grey, spherical conidia, rough with maturity.
<i>Penicillium notatum</i>	Ascomycetes	velvety or woolly colonies initially white and become green	Conidiophore green in colour, repeatedly branched, brushy head.
<i>Mucor sp.</i>	Zygomycetes	filamentous colonies, fluffy appearance, white in colour	Globular sporangia round black in colour that was supported by elevated column like columella. Non-septate or partially septate hyphae .
<i>Sclerotium rolfii</i>	Basidiomycetes	It was characterized as felty white appearance	Silky white hyphae tend to aggregate into rhizomorphic cord as well as characteristic sclerotium – fan shape was found.
<i>Fusarium oxysporum</i>	Ascomycetes	It was characterized as white to pink colony	Moist appearance, red with cottony and orange brown mycelium, with light brown exudates hyphen septet, small conidia in chain, sickle shaped long conidia were also observed.
<i>Rhizoctonia solani</i>	Basidiomycetes	Buff to black colony	The mycelium is generally hyaline, irregular in shape. Hyphae produce branches at right and acute angles to the main hyphae. specialized hyphae composed of monilioid cells, that fuse together to produce hard structure called sclerotia.

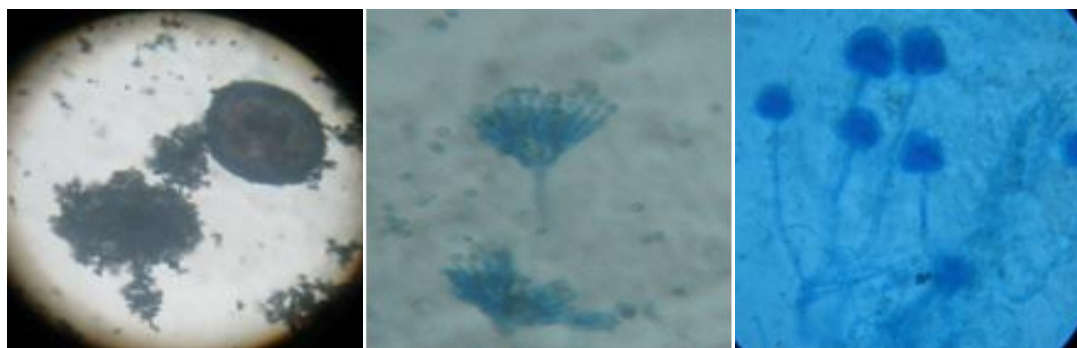
Fig.-I: *Aspergillus niger*Fig.-II: *Penicillium notatum*Fig.-III: *Mucor sp.*Fig.-IV: *Sclerotium rolfii* Fig.-V: *Fusarium oxysporum* Fig.-VI: *Rhizoctonia solani*

Plate- 2: Fig I – VI

On the basis of toxic potentiality of crude extracts two non- heterocystous (*Microcystis aeruginosa* & *Oscillatoria princeps*) and two heterocystous (*Nostoc calcicola* & *Anabaena anomala*) cyanobacterial isolates were selected for further experiment in view of the assessment of the antifungal effect of Cyanobacterial isolates on three Fungal isolates (*Fusarium oxysporum*, *Sclerotium rolfii* and *Rhizoctonia solani*). The data of percent mycelia inhibition and concerned graph indicated that the extract of *Microcystis aeruginosa* have great potential as fungicides carbendazim and Mancozeb compound against pathogenic fungi which was tested (Table – 3, plate -3; Fig. I - IV.). Toxic potentiality of *Nostoc calcicola* was found lesser than *Oscillatoria princeps* but greater than other cyanobacterial isolates, whereas minimum potentially of growth inhibition was observed in case of *Anabaena anomala*.

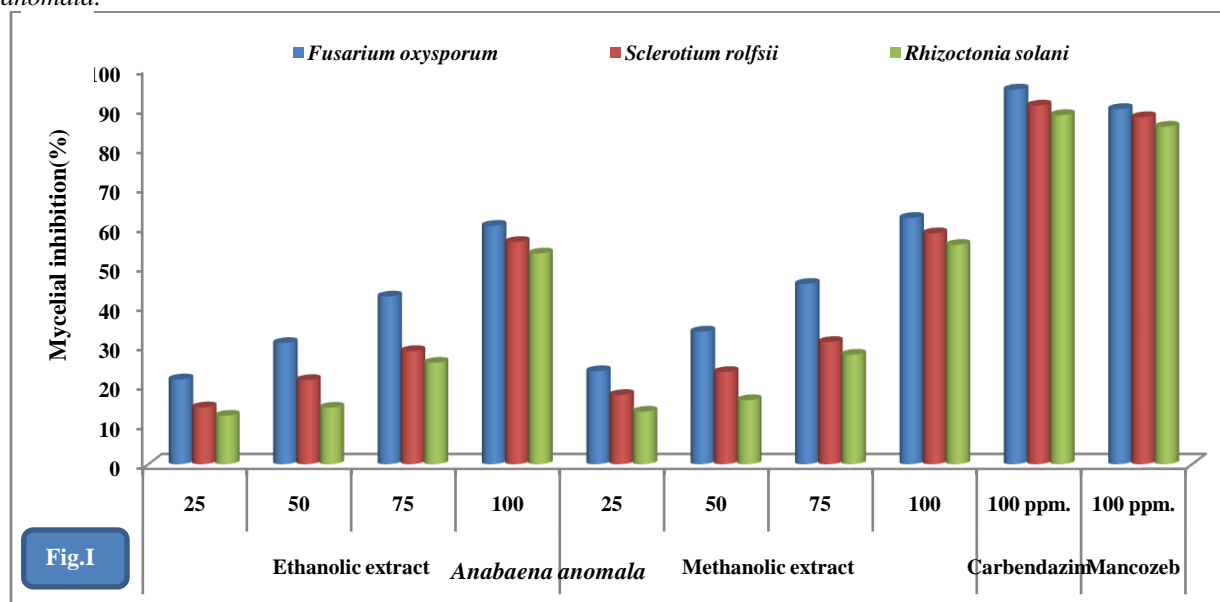
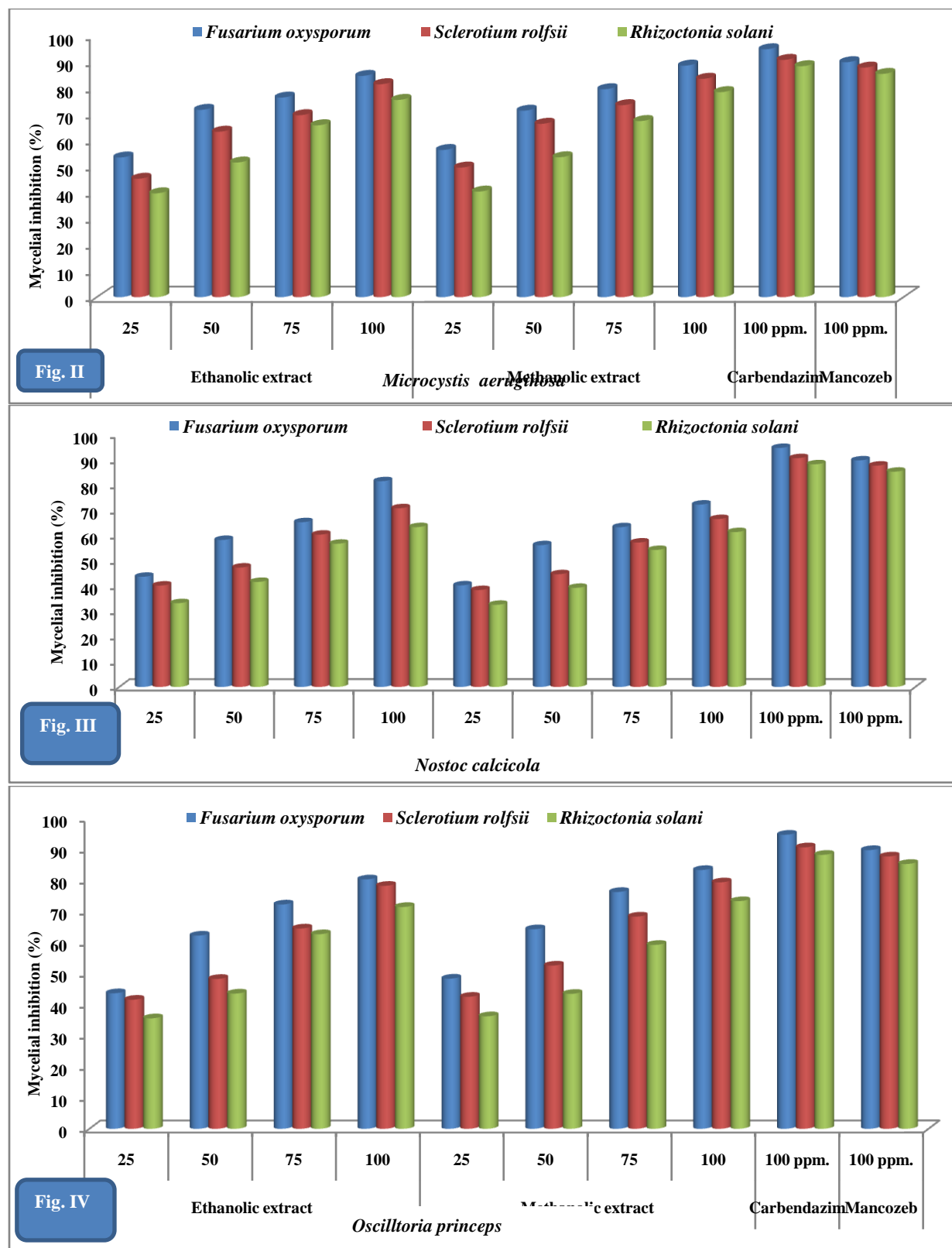


Fig.I



Cyanobacterial Isolates	Crude extracts	Conc. of crude extract	Percent of Mycelial Inhibition (Mean value \pm SD)		
			<i>Fusarium oxysporum</i>	<i>Sclerotium rolfsii</i>	<i>Rhizoctonia solani</i>
<i>Anabaena anomala</i>	Ethanol extract	25	21.42 \pm 0.25	14.35 \pm 0.18	12.23 \pm 0.22
		50	30.61 \pm 0.32	21.25 \pm 0.43	14.27 \pm 0.52
		75	42.37 \pm 0.27	28.58 \pm 0.26	25.62 \pm 0.35
		100	60.25 \pm 0.22	56.16 \pm 0.15	53.24 \pm 0.28
	Methanol extract	25	23.51 \pm 0.24	17.48 \pm 0.35	13.26 \pm 0.26
		50	33.52 \pm 0.22	23.28 \pm 0.43	16.17 \pm 0.52
		75	45.56 \pm 0.26	30.88 \pm 0.36	27.65 \pm 0.41
		100	62.25 \pm 0.33	58.35 \pm 0.38	55.41 \pm 0.29
<i>Microcystis aeruginosa</i>	Ethanol extract	25	53.37 \pm 0.32	45.25 \pm 0.24	39.67 \pm 0.25
		50	71.56 \pm 0.16	63.21 \pm 0.23	51.45 \pm 0.21
		75	76.24 \pm 0.34	69.51 \pm 0.28	65.66 \pm 0.19
		100	84.52 \pm 0.35	81.34 \pm 0.42	75.35 \pm 0.28
	Methanol extract	25	56.23 \pm 0.42	49.52 \pm 0.34	40.34 \pm 0.27
		50	71.24 \pm 0.36	66.23 \pm 0.45	53.45 \pm 0.28
		75	79.43 \pm 0.27	73.34 \pm 0.35	67.28 \pm 0.32
		100	88.46 \pm 0.24	83.42 \pm 0.28	78.33 \pm 0.45
<i>Nostoc calcicola</i>	Ethanol extract	25	43.65 \pm 0.35	40.18 \pm 0.43	33.21 \pm 0.56
		50	58.25 \pm 0.32	47.32 \pm 0.24	41.62 \pm 0.29
		75	65.28 \pm 0.32	60.35 \pm 0.46	56.75 \pm 0.34
		100	81.52 \pm 0.25	70.81 \pm 0.21	63.33 \pm 0.33
	Methanol extract	25	40.25 \pm 0.17	38.4 \pm 0.25	32.56 \pm 0.21
		50	56.18 \pm 0.42	44.67 \pm 0.16	39.28 \pm 0.32
		75	63.3 \pm 0.32	57.23 \pm 0.18	54.28 \pm 0.15
		100	72.26 \pm 0.26	66.53 \pm 0.34	61.36 \pm 0.42
<i>Oscillatoria princeps</i>	Ethanol extract	25	43.65 \pm 0.23	41.56 \pm 0.28	35.57 \pm 0.23
		50	62.21 \pm 0.35	48.26 \pm 0.27	43.55 \pm 0.26
		75	72.24 \pm 0.44	64.51 \pm 0.35	62.66 \pm 0.45
		100	80.32 \pm 0.35	78.24 \pm 0.42	71.44 \pm 0.28
	Methanol extract	25	48.35 \pm 0.15	42.51 \pm 0.35	36.23 \pm 0.22
		50	64.25 \pm 0.12	52.56 \pm 0.17	43.45 \pm 0.25
		75	76.27 \pm 0.27	68.33 \pm 0.35	59.23 \pm 0.32
		100	83.34 \pm 0.34	79.36 \pm 0.28	73.31 \pm 0.45
Solvent control		0	0	0	
Fungicides					
Carbendazim		100 ppm.	94.70 \pm 0.13	90.64 \pm 0.16	88.23 \pm 0.17
Mancozeb		100 ppm.	89.76 \pm 0.21	87.70 \pm 0.24	85.29 \pm 0.36

During present course of investigation, the results obtained regarding Cyanobacterial toxicity, revealed that the growth of all three fungal isolates were inhibited when subjected to crude extracts (Ethanol or Methanol) on solid nutrient media. Growth inhibition potency of crude extracts of cyanobacterial isolates have also been compared with standard fungicides against mycelial growth. The result of toxic potentiality as obtained against fungal pathogens has also been supported the findings. Cyanobacteria produce biologically active compounds that have allelopathic and toxic activity against plant pathogens [16][17]. Kim and Kim (2008) [18] reported inhibition of *F.oxysporum* f. sp. *lycopersici* by extracts of *N. commune* FA-103. Biological control of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) causing wilt disease of tomato was studied *in vitro* as well as under pot conditions and was concluded that *N. linkia*, can be utilised for the biological control of wilt disease of tomato which may help to obtain a higher yield and good health in agriculture [19]. Mohamed *et al.* (2011) investigated the suppression effect of cyanobacterial species- *Nostoc endophytum* and *Nostoc muscurum* against, the causal agent of Soya bean root rot *Rhizoctonia solani*.

The present research work correlates the findings of various workers as reported earlier regarding concerned research work. In this study Cynobacteria showed significant antifungal activities. This kind of investigation produces a much generalized view that Cynobacteria are capable of inhibiting growth of fungal pathogens. This work focuses on the ability of Canobacteria to be used as biocontrol agent further studies on Cyanobacterial

metabolites are essential for scientific workers to identify the toxic impact on phytopathogenic fungi which may lead to the formulation of significant bio-active compounds of biological origin, such as biofungicides.

Acknowledgment:-

Author is thankful to the Principal, Govt. E. Raghavendra Rao Postgraduate Science College, Bilaspur (C.G.) for providing research facilities and encouragement to carried out the present investigation.

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