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ANTIPHYTOPATHOGENIC POTENTIAL OF PYOCYANIN FROM Pseudomonas aeruginosa MH038270 AGAINST Fusarium oxysporum

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ABSTRACT:

Wide variety of economically important crops was affected by vascular wilt disease which is caused by Fusarium oxysporum. Wilt disease caused by F. oxysporum was one of the main reasons for reduction in yield of Vigna radiata (mung beans). The use of synthetic fungicides not only degrades environment and health but also lead to the development of fungicide resistant phytopathogens. To overcome these harmful effects, pyocyanin can be used as one of the alternatives. Pyocyanin pigment extracted from Pseudomonas aeruginosa MH038270 was examined for activities that inhibit the growth of phytopathogenic fungus in vitro. The antiphytopathogenic effect of pyocyanin was tested on the germination of F. oxysporum infected mung beans and purified pyocyanin had inhibitory effect on fungus. It was found that the seeds treated with pyocyanin showed better growth than the non-treated seeds.

Key words: - Pseudomonas aeruginosa; Pyocyanin; Fusarium oxysporum; Wilt disease; Antifungal; Vigna radiata.

INTRODUCTION:

Pulses are very important source of protein, minerals and vitamins and essential constituents of daily diet (Singh et al., 2015). There are various factors which affect the yield of pulses but diseases play an important role in its lower production. All around the world, pulses are infected by approximately hundred of fungal diseases. Among the diseases caused by fungus, vascular wilt caused by Fusarium is a major threat to pulses grower (Nelson, 1964; Ansari, 2003). It is a seed and soil borne disease. Many practices are there to minimize the disease such as chemical, biological, agronomic and use of resistant varieties (Mahmood et al., 2008). Natural compounds play important role in search of new fungicide (Cantrell et al., 2012). Microorganisms are biological agent which aid to solve many problems related to agriculture, environment and health (Satapute et al., 2012; Jogaiah et al., 2016; Satapute et al., 2019). There is growing interest in secondary metabolites produced by microbes because of their natural character, medicinal activities and safe to use feature. Pyocyanin pigment produced by Pseudomonas as secondary metabolite to protect itself from injurious effect had also several biological properties (Marrez and Mohamad, 2020). Pyocyanin pigment has the ability to arrest the electron transport chain of fungi and hence exhibit antifungal activity (Kerr et al., 1999). Additionally, pyocyanin is a broad spectrum pigment which inhibits pathogenic microbes, importantly on wilt disease which is occurred due to Fusarium oxysporum (Mahmoud et al., 2016). The biocontrol and antagonistic applications of pyocyanin pigment produced from P. aeruginosa is well documented (Jayaseelan et al., 2014). In the present research, the effect of aqueous pyocyanin from P. aeruginosa on mung bean plant infected with F. oxysporum was evaluated for better seed germination and enhanced crop production.

METHOD AND METHODOLOGY:

1.a Chemicals

All the chemicals used in this study were of analytical grade and procured from CDH and Hi-media, India. Media components used were of bacteriological grade.

1.b Microorganism

The pyocyanin pigment producing culture was isolated from clinical sample procured from IGMC, Shimla, India and identified as Pseudomonas aeruginosa MH038270 by 16s rRNA sequencing. The P. aeruginosa MH038270 was maintained on Nutrient agar medium (pH 7.0).

1.c Test microorganism

The antifungal effect of pyocyanin pigment was examined against Fusarium oxysporum MTCC 284. This fungus was purchased from Institute of Microbial Technology, Chandigarh, India. F. oxysporum was responsible for Fusarium wilt disease in mung beans.

2.a Preparation of purified fungicide solution

The pyocanin pigment was produced in the medium (pH 6.5) containing peptone 0.5 (%, w/v), beef extract 0.25 (%, w/v), NaCl 0.875 (%, w/v) and glycerol 2 (%, v/v), inoculated with 24h old inoculums and incubated at 37oC in orbital shaker (50rpm). The size of the inoculum was in accordance with the previously optimized value (3%, v/v) for maximum pyocyanin production. The fermentation broth was centrifuged at 10,000rpm for 10 min and the supernatant was used to extract pyocyanin by using chloroform extraction method. The extracted crude pigment was then purified by silica gel chromatography.

2.b Assay for quantification of pyocyanin

Pyocyanin was extracted from culture supernatant and measured based on the absorbance of pyocyanin in acidic solution at 520nm (Essar et al., 1990). The fermentation broth was centrifuged at 10000rpm for 10min. The culture supernatants were transferred into new test tubes and extracted with chloroform (1:2) and the aqueous phase was removed. The bottom layer was re-extracted with 1ml of 0.2N HCl until color change was observed. Following this, the absorbance of the pigment solution was measured using spectrophotometer at 520nm. The concentration of was calculated as microgram pyocyanin pigment produced per milliliter of culture supernatant. The optical density at 520nm was multiplied by 17.072 (extinction coffiecient) to determine the concentration of pigment (Sarkisova et al., 2005).

3.a Determination of antifungal activity of pyocyanin

Agar well diffusion technique is widely used to evaluate the antimicrobial effect of plants or microbial extracts (Magaldi et al., 2004; Valgas et al., 2007). Antifungal assay of purified filter sterilized pyocyanin pigment was performed by agar well diffusion method in Mueller Hinton Agar, Modified (MHA, Modified) plates containing 2% glucose with 0.0005g/L methylene blue (as per CLSI for antifungal). The plate was spread with standardized (0.5McFarland) fungal culture broth. Pyocyanin pigment of 100µg/mL concentration was prepared in chloroform. Each well of 6mm was filled with different concentration of pyocyanin. It was allowed to diffuse for about 30 minutes at room temperature and incubated for 18-24h at 35±2°C. The observed zone of inhibition (ZOI) was measured in mm.

3.b Effect of pyocyanin on germination and growth of Vigna radiata infected with F. oxysporum MTCC 284

The F. oxysporum infected mung seeds (72h) and soaked seeds were treated with purified aqueous pyocyanin (200µg/mL) for 2h extracted from P. aeruginosa to investigate its effect on growth of plant. The seeds were selected on the basis of shape, color, appearance and weight to eliminate the bad ones. After the proper treatment, selected seeds were sown at a suitable depth in organic

culturing soil in plastic tray with different segments. The planting of seed at a proper depth (10-12cm) can reduce the incidence of disease (Singh and Sandhu, 1973) whereas; shallow shown seed can be affected by various factors. The plastic tray was placed in green house for germination of seeds for 21 days. This experiment was planned as per scheme given below (Figure 1). The growth (above and below the soil) and weight of plant (fresh and dried) was measured in each case. The data was subjected to statistical analysis for calculating mean± SD.

RESULT & DISCUSSION

1.a Sensitivity of F. oxysporum MTCC 284 to pyocyanin produced by P. aeruginosa

Sensitivity of test microorganism was checked by measuring zone of inhibition against the pyocyanin at different concentrations except control. The purified pyocyanin was found to show antifungal activity against F. oxysporum MTCC 284 on modified Mueller Hinton Agar (Figure 2). The zone of inhibition was increase with increase in concentration of pigment. The maximum zone of inhibition was 34mm, which showed that this fungus was highly sensitive to pyocyanin (Table 1).

It has been reported that pyocyanin pigment induced triggering systemic resistance against Fusarium wilt of tomato (Audenaert et al., 2002). Pyocyanin also inhibited the growth of Aspergillus niger (Kerr, 1994), A. fumigatus and Candida albicans isolated from sputum of cystic fibrosis patients (Kerr et al., 1999).

1.b Effect of pyocyanin on the germination and growth of plant

The effect of pyocyanin pigment on the growth of mung seeds infected with phytopathogenic fungi, F. oxysporum MTCC 284 was studied and the results were represented in Figure 3. It was cleared from the results that pyocyanin pigment affects the growth of F. oxysporum. Pyocyanin treated seeds showed a good response for germination and growth with total plant height of 21.6±1.0cm and 0.5226±0.5g of fresh weight of plant (as shown in Table 2). The seeds infected with fungus have total plant height of only 14.5±1.66cm and fresh weight of 0.2769±0.6g, whereas in case of fungus infected seeds treated with pyocyanin showed a good growth. It shows that pyocyanin besides as an antifungal agent, also enhance the growth of plant when results were compared with control.

Pyocyanin pigment produced from Pseudomonas aeruginosa PUPa3 showed biocontrol activity against phytopathogenic fungi that infect tobacco, groundnut, rice, mango, chilli, sugarcane, tea, banana crops and cotton (Sunish et al., 2005). It has been also reported that pyocyanin produced from Pseudomonas species isolated from rhizosphere soil were used as biocontrol agent against Fusarium, the causative agent of Phythium damping of bean and wilt of chickpea (Anjaiah et al., 2003).

CONCLUSION

Our study showed that the pyocyanin pigment produced from P. aeruginosa exhibited a very potent antifungal activity against F. oxysporum, which is responsible for causing vascular wilt disease in plants. Pyocyanin pigment is also responsible for enhancement of growth in mung bean plant. As many investigations focus on agricultural bioactivities, our study suggest that exploring pyocyanin pigment as an antiphytopathogenic agent represent a promising alternative for discovering new non- toxic fungicide. Pyocyanin can be used in sustainable agriculture as a biocontrol agent against food spoilage and pathogenic fungi and bacteria.

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CONFLICT OF INTEREST:

The authors declare that there is no conflict of interest.

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Table 1: Zone of inhibition of *F. oxysporum* MTCC 284 against pyocyanin

Test Fungi	**Zone of inhibition at different concentration of pyocyanin**			
F.oxysporum MTCC284	**2µg**	**3µg**	**4µg**	**5µg**
	29±1.4	32±1.0	33±1.13	33.8±0.52

The values represented are mean ± SD, where n=3

Table 2: Growth of *Vigna radiata* plant

Group No.	**Experiment**	**Plant height (cm)**	**Shoot height (cm)**	**Root height (cm)**	**Leaf width (cm)**	**Leaf length (cm)**	**Fresh weight (g)**	**Dry weight (g)**

1	Soaked seeds sowed directly in soil (Positive control)	15.83±1.6	10.6±1.21	5.23±1.16	1.43±0.25	3.4±0.26	0.4294±0.05	0.0428±0.003
2	Seeds treated with pyocyanin for 2h	21.6±1.0	17.8±1.12	3.86±0.15	1.43±0.05	3.5±0.43	0.5226±0.05	.0624± 0.007
3	*F. oxysporum* MTCC 284 infected seeds	14.5±1.66	10.1±0.65	4.83±1.75	1.23±0.15	2.73±0.25	0.2769±0.06	0.0294±0.005
4	*F. oxysporum* MTCC 284 infected seeds treated with pyocyanin for 2h	17.6±0.75	13.7±0.85	3.86±0.35	1.36±0.11	3.9±0.36	0.5055±0.02	0.05956±0.005

The values represented are mean ± SD, where n=3

Figure 1: The experimental plan to determine the effect of pyocyanin on growth of seeds

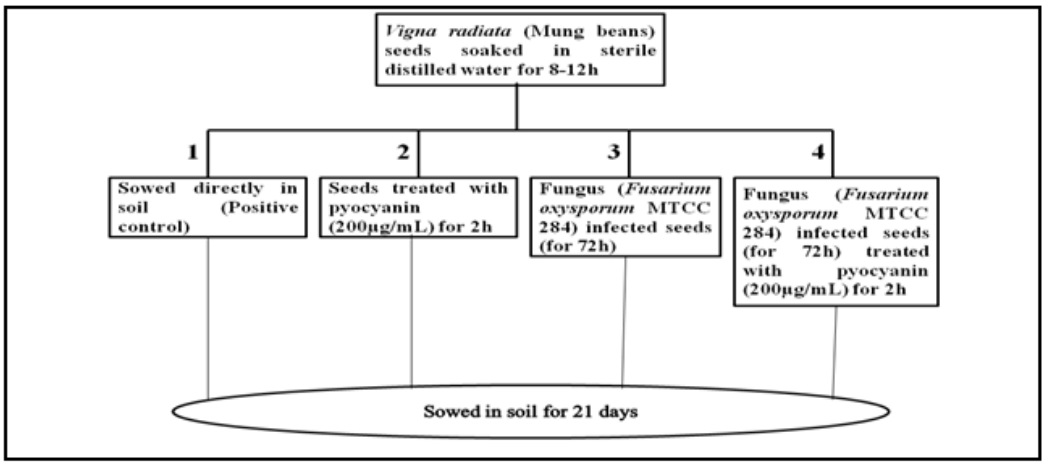

Figure 2: Zone of inhibition of *F. oxysporum* MTCC 284 at different concentration of pyocyanin.

Well 1: 2µg/20µL; 2: 3µg/30µL; 3: 4µg/40µL; 4: 5µg/50µL and centre: Negative control (contain only solvent).

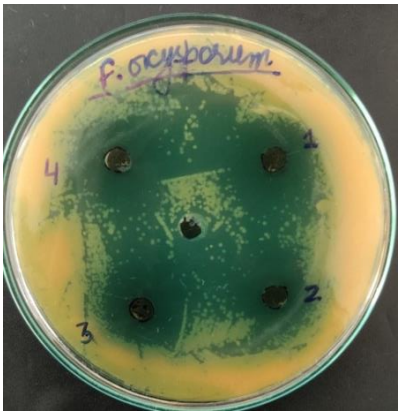

Figure 3: Growth of mung seeds (*Vigna radiata*)