EFFECTS OF PYOCYANIN PIGMENT ON THE CHEMICAL AND PHYSICAL CHARACTERISTICS OF AGRICULTURAL SOILS

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Abstract. The widespread use of agricultural chemicals, such as pesticides and fertilizers, has caused imbalances in agricultural soil. The use of several alternative methods (such as bio-fertilizers) has been suggested. Recent studies have focused on the application of microbial products instead of microbial cells to enhance plant growth. The present study aimed to investigate the effects of pyocyanin (produced as a secondary metabolite by *Pseudomonas aeruginosa*) on the chemical and physical properties of agricultural soil. Two plants, *Lens culinaris* and *Eruca sati*va, were utilized in the study. Based on the results, soil treatment with pyocyanin caused significant increase in some nutrients and minerals such as magnesium, chlorine, and iron, levels of which rose gradually with increasing pyocyanin concentration in the soil from both plants. In contrast, a gradual decline in levels of certain heavy metals, such as copper, lead, manganese, and boron, were observed, and the highest concentrations were reported in the control samples. Positive correlation was observed between electrical conductivity and hydrogen ion concentration in the soil, both of which decreased gradually with increasing pyocyanin concentration in the soil from both plants. Moreover, the *P. aeruginosa* strain studied herein showed significant antimicrobial activity against plant pathogenic fungi and bacteria. Overall, the study proved the positive interplay between pyocyanin and environmental factors that affect the development of plants, making it an ideal target for extended future research according to its environmental and agricultural significance.
Keywords: *Pseudomonas aeruginosa, bio-fertilizers, heavy metals, essential elements*

Introduction

Soil nutrients are essential for plant development and crop production because they control many plant processes. Mineral nutrition has also been identified as an important component in plant disease management and nutrient availability through soil modification (Moreira et al., 2015). While nutrients may be available in the soil, their chemical properties may hinder plants from using them. There are several techniques for supplying plants with essential nutrients, including chemical and biological approaches (Miransari et al., 2014).

Plant growth-enhancing microorganisms, such as rhizobia, mycorrhizae, and plant growth promoting bacteria, have been found to increase plant development under both stressed and non-stressed condition, influencing micronutrient solubility in the soil and subsequently, absorption thereof by plants (Lim et al., 2019). Although these bacteria are naturally found in the rhizosphere and plant tissue, they are frequently insufficient to provide the desired effects. Resultantly, it is recommended to isolate and prepare them as a microbial inoculum (Bender et al., 2016). This employment of microorganisms by inoculation is known as bio-fertilization, which can influence soil nutrient availability and hence, plant development (Hirel et al., 2011).

Many studies have examined the capacity of microbial cells to inhibit plant diseases and alleviate the effects of abiotic stress on plants, with encouraging results (Lim et al., 2019). Recent research has suggested that microbial compounds can be utilized successfully, rather than microbes that cannot survive in harsh environments and even if they do, their effectiveness may be diminished. Further, compounds are less susceptible

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to abiotic stress, and if the microorganisms are facultative pathogens, microbial compounds may be a preferable alternative (Compant et al., 2019).

Pseudomonas aeuginosa (*P. aeruginosa*); is a versatile bacterium found in a variety of aquatic and terrestrial habitats (Streeter and Katouli, 2016). Some strains are classified as rhizobacteria because they may colonize root surfaces and promote plant development (Anjaiah et al., 2003) whereas, other strains are capable of degrading environmental pollutants (Hasanuzzaman et al., 2004). It is also a notable opportunistic bacterium that may cause a range of diseases, including nosocomial infections (Del Barrio-Tofiñno et al., 2020). Additionally, it has been reported as an effective agent in a variety of applications, including bio-control (Anjaiah et al., 2003) and bioremediation programs (Vieto et al., 2021). Moreover, it may produce several extracellular secondary metabolites, including pyocyanin pigment; this blue-green, water-soluble, nitrogen-containing, heterocyclic phenazin has enjoyed special interest due to its capacity to generate reactive oxygen species (Hassani et al., 2012). Pyocyanin has been used in biosensors as a redox chemical for electron transfer between enzyme molecules and electrode materials; these biosensors are intended for use in different arenas, including the environment, agriculture, and medicine (Priyaja et al., 2014). It also plays a role in the control of phytopathogens (Sudhakar et al., 2013).

Most previous studies involved the use of microorganisms as bio-fertilizers. However, current research is tending towards investigation the potential of microbial products as important plant growth regulators. Recently, pyocyanin has received considerable attention due to its beneficial properties and applications in several different fields. Accordingly, the present study was designed to determine the effects of pyocyanin pigment on the chemical and physical properties of agricultural soil. Two plants, *Eruca sativa* and *Lens culinaris* which are known as arugula and lentil, respectively, were employed in obtaining the research results. These two plants were selected regarding to their high nutritional value and their widespread around the world as agricultural crops.

Materials and methods

Isolation and cultivation of bacteria

The *P. aeruginosa* strain used in this research was isolated and identified in a previous study (Al-Zahrani, 2012), based on the cultural, morphological, and biochemical characteristics described by Schaad et al. (2001). It was cultured and preserved at 4 °C on cetrimide slant agar.

Antimicrobial activity of bacteria

The antimicrobial activity of the *P. aeruginosa* strain was tested using the agar plate diffusion technique described by Lim et al. (2019), against the pathogenic microorganisms *Rhizoctonia solani, Fusarium oxysporium, Staphylococcus aureus and Escherichia coli.* Screening was performed in triplicate using potato-dextrose-agar (PDA) medium for the fungi and Muller-Hinton medium for the bacteria. Growth inhibition of bacteria and fungi was measured after 24 h and 6 d, respectively.

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Production of pyocyanin pigment

Selected colonies of *P. aeruginosa* were transferred from cetrimide agar to King's B broth medium to prepare the inoculums for pyocyanin pigment production, as described by Özyürek et al. (2016).

The effect of pyocyanin on soil

Digestion of soil samples

The soil that was used in this study is a mixture of peat-moss and sandy soil (2:1) obtained from the local market in Jeddah, in the west of Saudi Arabia. A known weight (0.5 g) of air-dried soil samples was used for digestion, following the procedure reported by Da-Silva (2013). Samples were placed in glass containers in the refrigerator at 4 °C, until use. All digestions were performed in triplicate.

Estimation of elements in the soil

Level of essential elements and heavy metals in digested dry soil samples, were estimated following the procedure reported by Cottenie et al. (1982). Additionally, hydrogen ion concentration (pH) and electrical conductivity (EC) were measured according to the procedure of Corwin and Yemoto (2020). All samples were analyzed thrice.

Soil treatment with pyocyanin

The effect of pyocyanin pigment on the chemical and physical properties of agricultural soil was studied using a crude solution of pigment, made up to different concentrations (5, 15, and 25%, respectively) by adding sterilized distilled water. *E. sativa* and *L. culinaris* seeds were grown in pots (30 diameter × 40 height) and irrigated every other day using pyocyanin solution. One month later, soil samples from the two plants were collected and analyzed to measure levels of essential elements, heavy metals, along with the pH and EC. All experiments were performed in triplicate.

Statistical analysis

All collected data were analyzed to determine the mean and standard deviation (SD) at $P \leq 0.05$, using SPSS software.

Results

Antimicrobial activity

The selected *Pseudomonas* strain was re-cultured on King's B medium and confirmed as *P. aeruginosa,* which was gram-negative, oxidase and catalase positive, did not ferment lactose, emitted a grape-like odor and produced blue-green pigment (*Fig. 1*).

The strain was tested for antimicrobial activity against different bacteria and fungi. The results are shown in *Table 1* and indicate antimicrobial activity against all tested microorganisms. The highest activity levels were reported against the fungi *R. solani* and *F. oxysporium,* with an average inhibition zone of 32 and 30 mm, respectively.

Antibacterial activity was monitored against both gram-positive and negative bacteria. The lowest activity level (inhibition zone diameter: 15 mm) was detected against *E. coli*,

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whereas higher activity was perceived against gram-positive *S. aureus*, with an average diameter of the inhibition zone, of 28 mm.

Figure 1. *Cultural characteristics of Pseudomonas aeruginosa and pyocyanin production on King's B agar, after 48 h at 37 °C*

Table 1. *Antimicrobial activity of Pseudomonas aeruginosa against tested microorganisms*

Tested microorganisms	**Inhibition zone diameter (mm) ± SD**
Rhizoctonia solani	32 ± 0. 03
Fusarium oxysporium	30 ± 0.00
Staphylococcus aureus	28 ± 0. 04
Escherichia coli	15 ± 0. 02

Essential elements in soils treated with pyocyanin

The results in *Tables 2* and *3* reveal the response of some essential elements (Mg, Ca, N, Cl, Na, K, and P), which are present in the growing soil of *L. culinaris* and *E. sativa*, before and after exposure to different concentrations of pyocyanin. It was observed that magnesium content in the soil increased gradually with increasing pyocyanin concentration, for both plants. The highest magnesium concentration observed were 46% for *L. culinaris* and 45% for *E. sativa*, at pyocyanin concentration of 25%.

Table 2. *Essential elements in Lens culinaris soil irrigated with different concentrations of pyocyanin*

Pyocyanin conc.	**Essential elements (%) ± SD**						
	Cl	Na	Mg	Ca	K	P	N
Control	317.9 ± 0.02	0.13 ± 0.01	0.36 ± 0.01	0.20± 0.01	0.10 ± 0.00	0.02 ± 0.01	0.10 ± 0.01
5%	320.4 ± 0.01	0.11 ± 0.03	0.40 ± 0.03	0.12 ± 0.02	0.11 ± 0.01	0.02 ± 0.01	0.09 ± 0.02
15%	324.2 ± 0.01	0.12 ± 0.01	0.44 ± 0.05	0.11 ± 0.04	0.11 ± 0.01	0.02 ± 0.02	0.08 ± 0.02
25%	341.4 ± 0.04	0.12 ± 0.07	0.46 ± 0.02	0.10 ± 0.06	0.10 ± 0.02	0.01 ± 0.04	0.05 ± 0.01

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Table 3. *Essential elements in Eruca sativa soil irrigated with different concentrations of pyocyanin*

Pyocyanin conc.	Essential elements (%) ± SD						
	Cl	Na	Mg	Ca	K	P	N
Control	248.5 ± 0.01	0.14 ± 0.01	0.30 ± 0.04	0.20 ± 0.02	0.10 ± 0.05	0.01 ± 0.01	0.15 ± 0.01
5%	267.5 ± 0.09	0.11 ± 0.03	0.36 ± 0.04	0.16 ± 0.05	0.10 ± 0.02	0.02± 0.02	0.13 ± 0.02
15%	280.5 ± 0.06	0.12 ± 0.01	0.38 ± 0.01	0.15 ± 0.02	0.11 ± 0.01	0.02 ± 0.00	0.09 ± 0.01
25%	284.4 ± 0.05	0.13 ± 0.03	0.45 ± 0.01	0.14 ± 0.00	0.12 ± 0.01	0.01 ± 0.01	0.06 ± 0.03

Similarly, soil treated with 25% pyocyanine exhibited the highest concentrations of chlorine, levels of which increased gradually in the soil of both plants, with increasing pyocyanin concentrations. Compared to the controls that reflected the lowest chlorine concentrations of 317.9% and 248.5% for *L. culinaris* and *E. sativa*, respectively. The highest concentrations were 314.4% for *L. culinaris* and 284.5% for *E. sativa* at 25% pyocyanin exposure.

In contrast, as pyocyanin concentration increased, the calcium and nitrogen content decreased. The lowest percentages of both elements were reported in soil treated with 25% pyocyanin, for both plants. No significant differences were observed in the levels of sodium, potassium, and phosphor in soil treated with different concentrations of pyocyanin, compared to the control, for either plants.

Heavy metals in soils treated with pyocyanin

The data in *Tables 4* and *5* reflect the heavy metal contents (Fe, Pb, B, Cu, Zn, and Mn) in the growing soil of *L. culinaris* and *E. sativa*, before and after treatment with different concentrations of pyocyanin. The iron concentration was highest in the soil samples before treatment, followed by manganese, while copper levels were the lowest. The results show different values of heavy metals in the soil when treated with different concentrations of pyocyanins, for both plants.

Table 4. *Heavy metals in Lens culinaris soil irrigated with different concentrations of pyocyanin*

Pyocyanin conc.	Heavy metals (ppm) ± SD					
	Pb	B	Cu	Zn	Mn	Fe
Control	27.7 ± 0.01	18.1 ± 0.00	13.6 ± 0.01	31.4 ± 0.02	105.4 ± 0.01	753.4 ± 0.02
5%	27.2 ± 0.02	15.1 ± 0.01	13.1 ± 0.01	42.9 ± 0.03	93.9 ± 0.01	760.7 ± 0.07
15%	22.3 ± 0.01	10.2 ± 0.06	12.0 ± 0.00	36.4 ± 0.05	92.3 ± 0.04	761.7 ± 0.03
25%	22.1 ± 0.04	5.5 ± 0.05	11.2 ± 0.01	35.5 ± 0.02	90.5 ± 0.02	774.7 ± 0.03

Table 5. *Heavy metals in Eruca sativa soil irrigated with different concentrations of pyocyanin*

Pyocyanin conc.	Heavy metals (ppm) ± SD					
	Pb	B	Cu	Zn	Mn	Fe
Control	24.7 ± 0.01	29.5 ± 0.01	15.3 ± 0.01	25.0 ± 0.01	100.1 ± 0.02	615.4 ± 0.05
5%	23.0 ± 0.01	18.5 ± 0.01	15.0 ± 0.02	30.6 ± 0.06	97.8 ± 0.02	761.7 ± 0.03
15%	21.4 ± 0.02	13.9 ± 0.05	13.0 ± 0.01	35.5 ± 0.05	87.8 ± 0.01	762.6 ± 0.07
25%	19.7 ± 0.02	10.4 ± 0.03	11.8 ± 0.04	33.5 ± 0.03	85.9 ± 0.05	775.7 ± 0.04

A gradual increase in iron level was observed with increasing pyocyanin, with the highest concentrations reported at 25% pyocyanin exposure; values were 774.7 ppm in *L. culinaris* soil and 775.7 ppm in *E. sativ*a soil, compared to the control which 753.4 and 615.4 ppm were recorded for the two plants as mentioned above, respectively. Concerning zinc, the results showed varying values, with the highest concentration in *L. culinaris* being 42.9 ppm at 5% pyocyanin, and the highest concentration in *E. sativeva* being 35.5 ppm at 15% pyocyanin.

The remaining heavy metals (lead, boron, copper, and manganese) concentrated in the control samples of growing soil of both plants, and gradual decreased with increasing pyocyanin concentration. The lowest values of all these elements were reported at 25% pyocyanin in the soil of both plants.

Soil electrical conductivity and pH

The EC of a soil solution is often used to determine the concentration of soluble salts in the soil. Herein, the EC of growing soil of *L. culinaris* and *E. sati*va was measured before and after treatment with different concentrations of pyocyanin. The results in *Table 6* show that the highest rate of EC appeared in the control samples for both plants. It is notable that the EC rate decreased gradually with increasing pyocyanin in treatment, whit the lowest values recorded as 0.56 and 0.44 dSm^{-1}, respectively at 25% pyocyanine.

Table 6. *Electrical conductivity of soil treated with different concentrations of pyocyanin*

Pyocyanin conc.	**Electrical conductivity (dSm^{-1}) ± SD**	
	Lens culinaris	*Eruca sativa*
Control	1.38 ± 0.03	1.84 ± 0.01
5%	1.24 ± 0.01	1.01 ± 0.01
15%	1.02 ± 0.02	0.74 ± 0.02
25%	0.56 ± 0.06	0.44 ± 0.04

Table 7 illustrates the soil pH values before and after treatment with different concentrations of pyocyanin. The results revealed that soil pH decreased gradually with increasing pyocyanin concentrations, with the lowest values recorded as 8.75 and 8.81, respectively, at 25% pyocyanin.

Table 7. *Hydrogen ion concentration of soil treated with different concentrations of pyocyanin*

Pyocyanin conc.	**Hydrogen ion concentration (pH) ± SD**	
	Lens culinaris	*Eruca sativa*
Control	9.12 ± 0.00	9.35 ± 0.01
5%	9.11 ± 0.01	9.20 ± 0.02
15%	8.81 ± 0.02	9.13 ± 0.04
25%	8.75 ± 0.02	8.81 ± 0.03

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Discussion

Antimicrobial activity

The relevant *P. aeruginosa* strain was tested for antimicrobial activity against different bacteria and fungi, as illustrated in *Table 1*. The results revealed higher inhibitory activity against fungi than bacteria. This finding supports the study outcomes of El-Fouly et al. (2015), who concluded that pyocyanin could modify and stop the electron transport chain in fungi, resulting in the formation of free oxygen radicals with antifungal properties. Moreover, Navarros et al. (2019) found that *Pseudomonas* species generate various phenazine chemicals with variable effectiveness against fungi.

Consistent with the findings of Özyürek et al. (2016), the present study showed that pyocyanin has a much stronger effect on gram-positive than gram-negative bacteria. According to a previous study by Das and Manefield (2012), variations in the lipid composition of the cell walls of gram-positive and negative bacteria may be responsible for this variance in pyocyanin sensitivity.

Essential elements in soils treated with pyocyanin

Tables 2 and *3* reflect the essential elements content of soil from both plants, treated with different concentrations of pyocyanin during this study. It is clearly shown that magnesium levels increased with increasing pyocyanin concentration. This result is significant since magnesium is a vital nutrient in plant physiology, as it is required for photosynthesis and involved in the chlorophyll structure (Moreira et al., 2015). Further, it can alleviate the symptoms of 22 different plant diseases (Huber and Jones, 2012).

The same observation was made regarding chlorine, which also increased with increasing pyocyanin concentrations in soil from both plants. According to several previous studies, chlorine is one of the elements of which large amounts can be detected in soil. It is involved in the regulation of cytoplasmic enzyme activities, turgor pressure and pH, along with photosynthesis, and contributes to stabilization of the membrane potential (Baetz et al., 2016). Chlorine deficiency is uncommon because its levels in soil are often high and plants only require minimal quantities (White and Broadlley, 2001). Moreover, it has been observed that chlorine homeostasis in plants is strongly connected to salt tolerance (Vatansever et al., 2016).

However, the percentages of calcium and nitrogen, were shown to decrease with increasing pyocyanin in soil from both plants. Previous studies suggest that nitrogen which is more mobile under humid circumstances is susceptible to processes such as leaching and denitrification, reducing its availability to the plant (Miransari et al., 2014).

In general, this study clearly indicated that pyocyanin influences the availability of several crucial nutrients and changes the soil characteristics. These findings are consistent with those of previous research, (Gupta et al., 2015; Naamala and Smith, 2020), which suggested that the addition of microbial products to soil as bio-fertilizer, could improve the availability and absorption of critical plant nutrients.

Heavy metals in soils treated with pyocyanin

Tables 4 and *5* illustrate the heavy metal content in the soil samples from both plants, before and after treatment with pyocyanin. A gradual rise in iron concentration was observed with increasing exposure to pyocyanin. This result may be explained by the findings of Cox (1986), which indicated that pyocyanin can be involved in solubilizing and releasing iron. Additionally, another study (Jayaseelan et al., 2013) found that pyocyanin is involved in iron metabolism and contributes to a process capable of reducing and releasing iron.

Zinc showed varying responses to different concentrations of pyocyanin, as illustrated by the results. According to Vatansever et al. (2016), zinc is a vital element for plant development and crop production, and it is influenced by soil microbial activity. Another study (He et al., 2010) indicated that zinc-tolerant bacterial strains such as *P. aeruginosa* can enhance zinc availability in soil and resultantly, its absorption by plants which is also important in bioremediation. The results also revealed a gradual decrease in the concentrations of other heavy metals (lead, boron, copper, and manganese) with increasing pyocyanin concentration. This observation clearly indicated that pyocyanin has a positive effect on reducing heavy metals and their toxic effects in soil, since heavy metal contamination is a significant issue of concern for natural ecosystems, in general. This finding agrees with majority of previous studies, observing that microbes have developed mechanisms to deal with metal toxicity, one of which is the production of different functional molecules (Teitzel et al., 2006; Hesse et al., 2018).

Soil electrical conductivity and pH

It is clearly shown in *Table 6* that the salt concentration in the soil decreased when treated with pyocyanin. This finding could be helpful in agriculture to reduce the problem of desertification resulting from the unsuitability of lands for cultivation, due to high salinity. Many previous studies, such as that by Corwin and Yemoto (2020), have proven the impact of salinity on plant production, which includes limiting plant water intake by lowering osmotic potential and altering soil permeability, making it more difficult for the plant to absorb water.

The soil pH was also measured in this study as shown in *Table 7*, a noticeable decrease in pH was observed in soil treated with pyocyanin. Soil acidity is a significant determinant of metal solubility and toxicity (Hesse et al., 2018). It has been demonstrated by Naamala and Smith (2020) that alkalinity affects and limits the availability of some metals, while toxicity is linked to low pH. Some studies have confirmed that the concentration of metals in, and the acidity of, soil are closely related to the types of microorganisms present in the soil and their production of certain extracellular microbial compounds (Hesse et al., 2018). Further, Miransari et al. (2014) proposed that some microbial products can alter the pH and activity of microorganisms in the rhizosphere, thereby influencing soil nutrient availability.

Conclusion

The results of this study revealed the effects of pyocyanin on the nutrients and minerals in soil. It was observed that levels of some important elements such as magnesium, chlorine, and iron had gradually increased when soil was treated with pyocyanin; the highest concentrations of all mentioned elements were recorded at a 25% pyocyanin concentration. In contrast, levels of some elements, such as calcium and nitrogen, had gradually decreased in the soil treated with pyocyanin, whereas, some elements were not affected. The gradual decrease in levels of certain heavy metals, in soil when treated with different concentrations of pyocyanin, was especially noteworthy. It is remarkable, that the gradual decrease in EC and soil pH after treatment with different concentrations of pyocyanin, can improve the properties of soil and make it more suitable for agricultural use. Moreover, the study has shown the ability of pyocyanin-producing bacteria to

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suppress plant pathogenic fungi and bacteria. The antimicrobial effect is stronger against fungi than bacteria, on the one hand, and against gram-positive over negative bacteria, on the other. The findings of this study have important implications for the application of pyocyanin to agricultural soil, which is of economic and environmental importance. Further research is required on the different products of the phytomicrobiome, such as pyocyanin, and their potential effects in reducing the adverse conditions that could affect plant growth, both under laboratory conditions and in the field.

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