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ENVIRONMENTAL INFLUENCE OF CULTURAL MEDIUM ON BIOHERBICIDAL ACTIVITIES OF *PSEUDOMONAS AERUGINOSA* C1501 ON MONO AND DICO WEEDS

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Key words: weed management, submerged fermentation, carbon source, mineral salts, bio--herbicides.

Abstract

Microbe producing natural herbicides are alternatives to the chemical herbicidal formulations. The effect of minerals and carbon sources were screened to select the best when combined and when apply singly during submerged fermentation. The effect of their phytotoxic metabolites was tested on *Chromolaena odorata and Echinochola crus-galli.*

It was observed that the best combination between all the mineral was found in the combination containing manganese, zinc, bromine and iron. It gave the highest bio-herbicidal activities on the tested weeds when compared with the basal medium without any mineral amendment ($P \leq 0.05$). The best carbon source screened was glucose while the best mineral screened was iron in term of showing activities on the tested weeds ($P \leq 0.05$).

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WPŁYW ŚRODOWISKA HODOWLANEGO NA AKTYWNOŚĆ FITOTOKSYCZNĄ *PSEUDOMONAS AERUGINOSA* C1501 W ZWALCZANIU CHWASTÓW JEDNO- I DWULIŚCIENNYCH

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S ł o w a k l u c z o w e: gospodarka chwastami, fermentacja, źródło węgla, sole mineralne, bio-herbicydy.

A b s t r a k t

Mikroorganizmy wytwarzające naturalne herbicydy są alternatywą dla chemicznych preparatów chwastobójczych. Badano wpływ soli mineralnych i źródeł węgla na wydajność fermentacji podczas osobnego i łącznego ich stosowania. Wpływ fitotoksyczności uzyskanych metabolitów testowano na *Chromolaena odorata* i *Echinochloa crus-galli*.

Wykazano, że spośród wszystkich badanych pierwiastków kombinacja zawierająca mangan, cynk, brom i żelazo dała najwyższą biologiczną aktywność chwastobójczą w porównaniu z podstawową pożywką bez zmiany składu mineralnego ($P \leq 0{,}05$). Pod względem aktywności fitotoksycznej najlepszym źródłem węgla była glukoza, a najlepszym pierwiastkiem żelazo ($P \leq 0{,}05$).

Introduction

Weeds are unwanted plants that compete directly and indirectly with economically important crops, reducing their yield, interfering with harvesting operations and provides hosts for insect pests and pathogens, as well as affecting the quality of the harvested product and thereby affecting the agricultural productivity of farmers in agricultural environments. This is one of the major reason mitigating against reduction in food production, in many parts of the world (BARRETO 2009, LORENZI 2000, ADETUNJI 2015).

The continuous usage of certain herbicides, or herbicides with similar mechanisms of action, in the same environment has led to the selection of weed populations that are resistant to certain chemical groups and becomes difficult to eradicate (OLIVEIRA JÚNIOR and INOUE 2011). Intensive and indiscriminate use of chemical pesticides might also lead to an ecosystem imbalance. This therefore necessitate the need for the use of biological agents for weed control particularly attractive (FONTES 1992, ADETUNJI and OLOKE 2013), following a global demand for an alternative control systems that are simultaneously effective, economic and less harmful to the environment.

It has been discovered that the active ingredient from most microorganisms are extracellular-secondary me-tabolites which are normally produced in culture me-dia serve as intermediates from primary metab-olisms as precursors for their biosynthetic process and they have various application as herbicides, anticancer agents, drugs, immunoregulators and antiparasitic agents. The environmental factors like as temperature and pH nutritional sources like carbon, nitro-gen, time, and minerals, have been discovered to have a profound influence on the activities of the active metabolite produced. Optimization of the culture conditions is essential to get high yields of the metabolites (SANCHEZ and DEMAIN 2002).

Therefore, this work intends to screen the best optimum mineral salt and carbon source when combine and apply singly that can influence the highest bio-herbicidal activities on the *Chromolaena odorata and Echinochola crus-galli* weeds.

Materials and Methods

Microorganisms and growth conditions

C1501 strain was isolated from the rhizosphere of wheat plants planted at the research farm of Nigerian Stored Product Research Institute, Ilorin Kwara State. The isolated bacteria was identified as *P. aeruginosa* C1501 with an accession number KF976394. The bacteria plates were incubated at 37°C for 48 h on Kings agar in BOD incubator. At the end of each incubation period, the colonies were subcultured onto fresh media maintained on slants of Kings agar and stored at 4°C in the refrigerator.

Optimization of *P. aeruginosa*

Bacteria were stored in 0.8% nutrient broth plus 0.5% yeast extract (NBY) broth (Difco, Detroit, Mich.) plus 40% glycerol at -80°C. Starter cultures were grown in 10-ml dilute (1/10-strength) NBY broth in 20-ml screw top vials for 8 to 12 h at 27°C at 140 rpm, yielding approximately 10^5 CFU ml^{-1}. Test cultures of 20 ml of NB or NBY broth (unbuffered) in 100-ml Erlenmeyer flasks were inoculated with 10 µl of starter culture. Chemical analysis indicated that NBY broth contained (mg l^{-1}): total nitrogen, 1441.0; amino nitrogen, 604.0; total phosphate, 600.1; potassium, 597.9; sodium, 259.7; chloride, 121.7; sulfate, 54.9; magnesium, 22.9; calcium, 6.1; zinc, 0.5; and boron, cobalt, copper, iron, lithium, manganese and molybdenum, < 0.1. The

sterile autoclaved medium was amended with filter-sterilized mineral solutions to give 1 mM $H^{33}O_3$, $CaCl_2 \cdot 2H_2O$, $FeSO_4 \cdot 7H_2O$, LiCl, $MgSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $Mo_7(NH_4)_6O_{24} \cdot 4H_2O$, NaCl, 0.7 mM $CuSO_4$, $ZnSO_4 \cdot 7H_2O$, or 0.1 mM $CoCl_2 \cdot 6H_2O$ and with sterile stock solutions of carbon sources to give 1% (wt/vol). Cultures were incubated for 48 h at 24°C with shaking at 140 rpm in darkness, unless otherwise indicated. Culture pH for all media was 6.5 to 6.7 at inoculation and 7.7 to 7.9 after 48 h of bacterial growth. Bacterial growth after 48 h was approximately 10^8 CFU ml^{-1} in NBY.

In other experiments, combinations of the following minerals were investigated:

1. $ZnSO_4 + H_3BO_3 + FeSO_4 + MnCl_2$.
2. $ZnSO_4 + H_3BO_3 + FeSO_4 + (NH_4)_6Mo_7O_{24}$.
3. $ZnSO_4 + FeSO_4$.
4. $ZnSO_4 + FeSO_4 + (NH_4)_6Mo_7O_{24}$.

All cultures were provided with 134 μm EDTA as a chelating agent. Flask cultures were inoculated using 1 ml of a 24 h NBY adjusted to 10^6 CFU ml^{-1} in sterile distilled water. Cultures were incubated for 48 h at 130 rpm and 26°C in a rotary shaker incubator, thereafter cell density was measured at 600 nm and cultures were used for bioherbicidal assay. All glassware, including flasks, were acid-washed with 0.1% HCl solution to remove residual minerals. Their combined effect on the phytotoxic metabolite was later determined on sterilized leaves of monocotyledonous and dicotyledonous weeds. They were later transferred to Petri plate containing moistened cotton ball and filter paper. The sterilized leaves were then inoculated with cell free cultural filtrate containing 100 μl of the different combinations while NBY serves as control with the help of sterile needle on the surface of the leaf. Later, plates were incubated at 25°C for one week. A daily observation was made for the development of necrotic lesions from the inoculated leaves (SLININGER et al. 1996).

Effect of minerals and carbon source on the production of phytotoxic metabolites from *P. areuginosa*

P. areuginosa was grown for 48 h in 20 ml portions of nutrient broth yeast medium, 8 different NBY plus different carbon sources (maltose, glucose, sorbitol, mannitol, dulcitol, rhamnose, sucrose, glycerol) containing 1% (wt/vol) of the various media were screened so as to determine the best carbon source. The best carbon source was later combined with $FeSO_4$, $ZnSO_4$, H_3BO_3 and $(NH_4)_6Mo_7O_{24}$. Cultures were incubated for 48 h at 130 rpm and 26°C in a rotary shaker incubator, thereafter the cell density was measured at 600 nm.

The bioherbicidal assay was later carried out with the phytotoxic metabolite produced from the combination of the different minerals with the best carbon source on sterilized leaves of monocotyledon and diacotyledon weeds as described above (SLININGER et al. 1996).

Data analysis

The data were analyzed by using SAS software 8.2 (2001). Significant means were separated using Duncan's multiple range test.

The graphical scheme of experiments is shown in Figure 1.

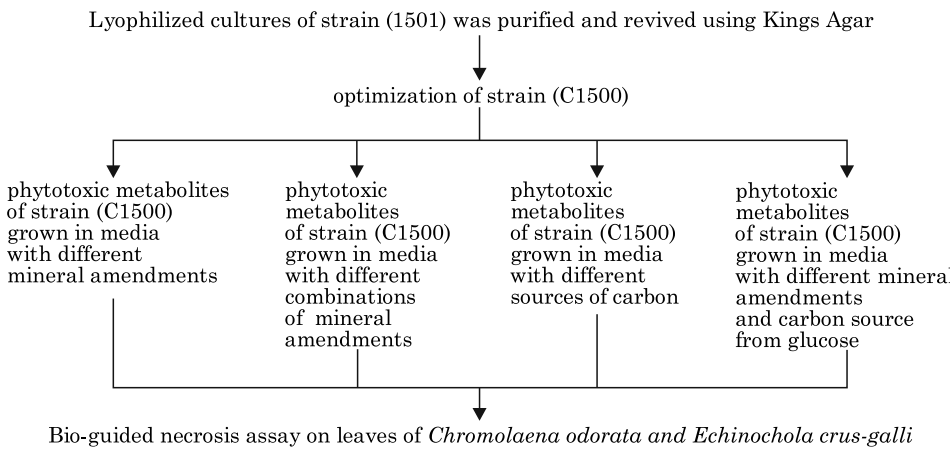

Fig. 1. Graphical scheme of experiments

Result

Optimization of *P. aeruginosa*

The phytotoxic activity of *P. aeruginosa* grown in media with different salt amendments was tested on *Chromolaena odorata and Echinochola crus-galli* leaves respectively. It was observed among all the tested minerals $FeSO_4$ amended with the basal medium produced the highest OD of 2.99 compared to the basal medium that had an OD of 1.63. The phytotoxic metabolite produced by *P. aeruginosa* induced a necrotic area of 3.5 mm^2 and 2.7 mm^2 ($P \leq 0.05$) compared to the basal medium without mineral amended that induced a necrotic area of 1.5 mm^2 and 1.0 mm^2 ($P \leq 0.05$) on *Chromolaena odorata and Echinochola crus-galli* leaves respectively (Table 1). It was observed that the

Table 1
Phytotoxic activity of *P. aeruginosa* grown in media with different mineral amendments on *Chromolaena odorata and Echinochola crus-galli*

Mineral amendments*	OD_{600}	Diameter of necrosis [mm]**	
		Chromolaena odorata	*Echinochola crus-galli*
Nutrient broth plus 0.5% yeast extract (NBY)	1.63 ± 0.7^{f}	0.9 ± 0.2^{e}	0.6 ± 0.21^{f}
Cacl	1.72 ± 0.31^{de}	1.5 ± 0.4^{de}	0.8 ± 0.1^{ef}
$MnCl_2$	2.95 ± 0.6^{a}	3.0 ± 0.6^{ab}	2.4 ± 0.3^{ab}
$CoCl_2$	1.78 ± 0.82^{d}	1.8 ± 0.2^{cde}	1.2 ± 0.5^{def}
H_3BO_3	2.31 ± 0.9^{c}	2.3 ± 0.25^{bcd}	1.9 ± 0.31^{abcd}
$FeSO_4$	2.99 ± 0.5^{a}	3.5 ± 0.7^{a}	2.7 ± 0.1^{a}
$ZnSO_4$	2.83 ± 0.4^{b}	2.8 ± 0.6^{abc}	2.1 ± 0.4^{abc}
$CuSO_4$	1.64 ± 0.9^{ef}	1.9 ± 0.2^{cde}	1.4 ± 0.6^{cdef}
$(NH_4)_6Mo_7O_{24}$	2.24 ± 0.52^{c}	2.0 ± 0.1^{bcde}	1.6 ± 0.4^{bcde}
LiCl	1.73 ± 0.7^{d}	1.7 ± 0.3^{cde}	0.9 ± 0.2^{ef}
NaCl	1.71 ± 0.6^{def}	1.6 ± 0.7^{de}	0.7 ± 0.1^{f}

Explanation: *medium without mineral amendments; **width of the diameter of necrosis on the eleaves. Means with different superscripts within the same column were not significantly different at 5%. Values are means ± standard error

best combination between all the mineral was found in the combination containing $ZnSO_4$ + H_3BO_3 + $FeSO_4$ + $MnCl_2$ with an OD of 3.21 compared to the basal medium without any mineral amendment with an OD of 1.93. The phytotoxic metabolite produced by *P. aeruginosa* induced a necrotic area of 3.8 mm^2 and 3.0 mm^2 ($P \leq 0.05$) compared to the basal medium without mineral amended that induced a necrotic area of 0.9 mm^2 and 0.6 mm^2 ($P \leq 0.05$) on *Chromolaena odorata and Echinochola crus-galli* leaves respectively (Table 2).

Different carbon sources were then screened to select the best carbon sources among all the tested sugars that produced the highest amount of metabolites. It was observed that when *P. aeruginosa* was inoculated into the different carbon sources, glucose produced the highest amount of phytotoxic metabolite with an OD of 3.0 while the basal medium without any mineral amendment with an OD of 0.7 (Figure 2*a*). The phytotoxic metabolite produced after the inoculation of *P. aeruginosa* into different carbon sources amended with different sugars showed that sucrose among all the sugars induced a necrotic area of 2.8 mm^2 and 2.4 mm^2 ($P \leq 0.05$) on *Chromolaena odorata and Echinochola crus-galli* leaves respectively (Figure 2*b*).

When the best carbon source screened was then combined with the best mineral salt, it was discovered that the combination containing $FeSO_4$ + glucose had an OD of 3.51 compared to the basal medium without any mineral amendment with an OD of 1.93. The phytotoxic metabolite produced from the medium that contained sucrose induced a necrotic area of 3.6 mm^2 and

Table 2
Phytotoxic activity of *P. aeruginosa* grown in media with different combinations of mineral amendments on *Chromolaena odorata and Echinochola crus-galli*

Mineral amendments*	OD_{600}	Diameter of necrosis [mm]**	
		Chromolaena odorata	*Echinochola crus-galli*
Nutrient broth plus 0.5% yeast extract (NBY)	1.93 ± 0.8^{b}	1.5 ± 0.4^{c}	1.0 ± 0.2^{b}
$ZnSO_4 + H_3BO_3 + FeSO_4 + MnCl_2$	3.21 ± 0.65^{a}	3.8 ± 0.5^{b}	3.0 ± 0.8^{a}
$ZnSO_4 + H_3BO_3 + FeSO_4 + (NH_4)_6Mo_7O_{24}$	3.19 ± 0.6^{a}	3.7 ± 0.2^{a}	2.9 ± 0.2^{a}
$ZnSO_4 + FeSO_4$	2.98 ± 0.7^{a}	3.6 ± 0.4^{ab}	2.7 ± 0.7^{a}
$ZnSO_4 + FeSO_4 + (NH_4)_6Mo_7O_{24}$	2.8 ± 0.4^{a}	2.5 ± 0.62^{bc}	2.1 ± 0.3^{ab}

Explanation: *medium without mineral amendments;**width of the diameter of necrosis on the leaves. Means with different superscripts within the same column were not significantly different at 5%. Values are means ± standard error

Table 3
Phytotoxic activity of *P. aeruginosa* grown in media with different mineral amendments and carbon source from glucose on *Chromolaena odorata and Echinochola crus-galli*

Mineral amendments*	OD_{600}	Diameter of necrosis [mm]**	
		Chromolaena odorata	*Echinochola crus-galli*
Nutrient broth plus 0.5% yeast extract (NBY)	1.93 ± 0.37^{d}	1.6 ± 0.2^{c}	0.9 ± 0.6^{d}
$FeSO_4$ + glucose	3.51 ± 0.83^{a}	3.6 ± 0.33^{a}	3.1 ± 0.2^{a}
$ZnSO_4$+ glucose	3.21 ± 0.4^{b}	3.21 ± 0.6^{ab}	2.53 ± 0.7^{b}
$(NH_4)_6Mo_7O_{24}$ + glucose	2.13 ± 0.6^{c}	2.83 ± 0.4^{ab}	2.10 ± 0.3^{c}
H_3BO_3 + glucose	2.02 ± 0.3^{cd}	2.42 ± 0.1^{b}	1.93 ± 0.5^{c}

Explanation: *medium without mineral amendments;**width of the diameter of necrosis on the leaves. Means with different superscripts within the same column were not significantly different at 5%. Values are means ± standard error

3.1 mm^2 ($P \leq 0.05$) on *Chromolaena odorata and Echinochola crus-galli* leaves respectively compared to basal medium that had 1.6 mm^2 and 0.9 mm^2 ($P \leq 0.05$) on *Chromolaena odorata and Echinochola crus-galli* leaves respectively (Table 3).

Discussion

Using microorganisms as a bioherbicide are uniquely capable of reducing invasive weed populations through highly specific impacts that are self-sustaining, contributing to the protection of natural ecosystems (DRIESCHE VAN et al. 2010). One group of microorganisms largely overlooked as biocontrol agents of weeds is the Deleterious Rhizobacteria (DRB) that can colonize plant root surfaces and able to suppress plant growth. A major group of rhizobacteria with potential for biological control is the *Pseudomonads*

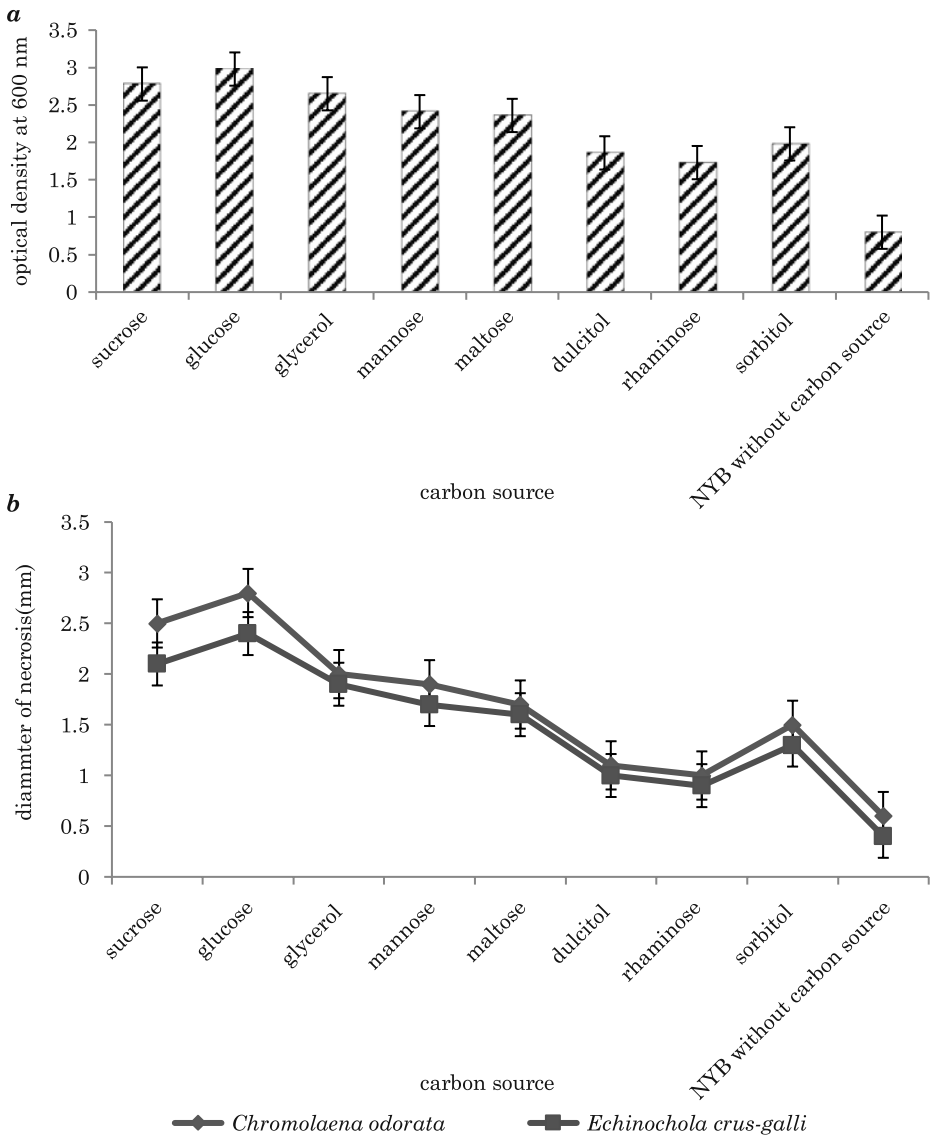

Fig. 2. Effect of: *a* – different sources of carbon on Optical density of P. *aeruginosa; b* – phytotoxic metabolites of *P. aeruginosa* produced from different sources of carbon on *Chromolaena odorata* and *Echinochola crus-galli*

(KENNEDY et al. 1991). During this study, *Pseudomonas aeruginosa* an example of the Deleterious Rhizobacteria (DRB) from Pseudomonades was used.

Optimization of media is generally done by studying the effects of the ingredients/nutrients on growth using fermentation studies, selecting and optimizing a few parameters (GRESHAM and INAMINE 1986). Nutritional factors such as carbon sources, nitrogen sources, trace metals, vitamins, carbon loading, and carbon-to-nitrogen ratio can all have an influence on growth, propagule formation, and biocontrol efficacy (JONSBU et al. 2002). Once an optimized defined medium has been developed, a production medium can be formulated by replacing the nutritional components of the defined medium with low-cost, complex substrates. Use of this directed optimization strategy not only aids in the development of production media for specific bioherbicides but also provides nutritional information which will be useful in developing production media for other microbial biocontrol agents. There are reports that medium and conditions used for production of biocontrol agents, influence their ability to survive during the formulation process (ZHANG et al. 2005). For example, mild thermal and pH stresses and carbon starvation can increase the resistance of cells to further stresses (OVERBEEK VAN et al. 1995).

Different mineral was amended with the basal medium in a submerged fermentation to screen out the best medium that produced the highest amount of phytotoxic metabolite on the tested weeds. It was observed that $FeSO_4$ and $ZnSO_4$ produced the highest colony forming unit and the largest necrotic area compared with the other mineral screened. Bacteria are usually mass-produced using liquid fermentation systems, but can also be produced through semisolid or solid-state fermentation (BOYETCHKO et al. 1999). Important conditions that must be considered are oxygen transfer, incubation temperature, nutrient requirement and agitation to ensure a large, stable, and efficacious bacterial population. The nutrients added to the medium should be inexpensive, readily available and conducive to a high biomass and proper secondary metabolite production (HYNES and BOYETCHKO 2006).

The combination of the basal medium with zinc in combination with molybdenum and iron improved the phytotoxic metabolite of *P. aeruginosa* after fermentation. There are some reports that zinc improves production of the antibiotics phenazine (OWNLEY et al. 2003, SLININGER and JACKSON 1992) and 2,4-diacetylphloroglucinol (DUFFY and DÉFAGO 1997, 1999).

It was observed that P. *aeruginosa* isolated from rhizospheres was able to produce a phytotoxic metabolite that can induce necrotic area on the leaves of tested weeds using submerge fermentation. KREMER et al. (1990) who reported that specific rhizobacteria which suppress weed growth are ubiquitous and probably found in all plant rhizospheres. Other examples of rhizosphere bacteria with bioherbicidal activity are *Enterobacter*, *Arthrobacter* and *Pseudomonas cichorii* (BOYETCHKO et al. 2002). This group of bacteria probably cause damage to the weed plant through the production of phytotoxins

that are taken up through the plant roots (KREMER et al. 1990). It has been reported that over 90% of fluorescent bacteria found in citrus root systems possess siderophore activity, which are likely involved in the suppression of weed growth (KREMER et al. 1990). In laboratory studies, over 100 bacterial isolates have been found to suppress the growth of grass weed roots by 80% (DAIGLE et al. 2002). According to KREMER et al. (1990), rhizobacteria will be successful in suppressing weed growth if they have a high colonizing ability, produce specific phytotoxin(s) that suppress growth of the host weed which are not suppressed by siderophores or antibiotics produced by competing microorganisms and have the ability to synthesize siderophores.

Green foxtail is another annual grassy weed, which is a weed of corn, soybean, cereals, canola, sugar beet, and pastures (DAIGLE et al. 2002). The suppression of weeds by *P. fluorescens* BRG100 has been attributed to secondary metabolites and phytotoxins. As mentioned previously, *Pseudomonas* spp. possess the ability to produce a variety of metabolites. This includes phytotoxins that cause symptoms such as root discolouration and reduced root length and also affect lipid synthesis and membrane integrity (BOYETCHKO et al. 2002).

It was observed that among all the carbon sources screened glucose followed by sucrose were able to produce the highest amount of colony forming unit and phytotoxic metabolites when *P. aeruginosa* was inoculated into the basal medium containing different carbon sources. Glucose is one of the primary molecules which serve as energy sources for almost all organisms, including bacteria. However, one of the most common growth media used in microbiology labs, nutrient broth, does not contain glucose as the main carbon source for bacteria (it contains protein). The addition of glucose to nutrient broth may increase the overall growth rates and biomass of bacteria over time. If so, this could be beneficial for lab purposes in that less time would be needed to grow cultures for experiments (NEIDHARDT et al. 1990). The result obtained during this study showed that glucose was a better carbon source is in line with NAMPOOTHIRI and PANDEY (1995) who showed that glucose gave best cell growth out of six carbon sources tested on *Brevibacterium* sp. in growth medium, EMANUILOVA and KAMBOUROVA (1992) who studied the effect of five carbon sources on *Bacillus steorothermophilus* found that the organism showed a preferential choice of growth and activity when grown on glucose as sole carbon source.

Conclusion

This study has shown that environmental factors had effect on the production of phytotoxic metabolite for the management of weeds from *P. aeruginosa* C1501 a potential bioherbicidal agent which could be an alternative

to the chemical herbicides. It was observed that when they were combined the highest bio-herbicidal activities was observed on the tested weeds when compared to the basal medium without any mineral amendment. Other studies to elucidate the active compounds still need to be carried out using from the metabolites, their mode of action, non-target effect and host range test of the active compound needs to be carried out as well as the greenhouse and field trial when compared with a chemical control. Therefore, the production of bioherbicides from this strain could be an alternative to the chemical herbicides which is not health and environmental friendly. We believe this work will be of great benefit and provide useful information for wide audience for so many sectors like for Industry, Environment and Agriculture. Also, it will provides vital information for entrepreneurs in business set up as well as farmers.

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