Modern Applied Science; Vol. 11, No. 10; 2017
ISSN 1913-1844 E-ISSN 1913-1852
Published by Canadian Center of Science and Education

Potency of *Bacillus spp* from Potato Rhizosphere as Active Ingredients for Biostimulant Formulation

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Received: August 9, 2017 Accepted: September 11, 2017 Online Published: September 27, 2017

doi:10.5539/mas.v11n10p74 URL: https://doi.org/10.5539/mas.v11n10p74

Abstract

Bacteria that colonized plant roots can be identify, characterify and tested for their ability to stimulate plant growth. The purpose of this research is to know the ability of *Bacillus spp* bacteria as active ingredient of biostimulant formulation which aims to stimulate the growth of plants. *Bacillus spp* bacteria derived from rooting potato plants tested its ability to dissolve phosphate, N fixation, producing IAA, GA, siderophore and HCN. The results showed different abilities in producing growth-promoting compounds from each tested strain. *Bacillus niabensis* Strain PT-32-1, *Bacillus subtilis* Strain SWI16b, *Bacillus mojavensis* Strain JCEN3, *Bacillus subtilis* Strain HPC21, *Bacillus cereus* Strain HY, and *Bacillus moyavensis* UCMB 5075 can be utilized as an active ingredient formulation biostimulant plant growth promoting non pathogenic.

Keywords: *Bacillus spp*, Biostimulant, Formulation, Non pathogenic, Potato

1. Introduction

Biostimulant products from bacterial active formulations are now widely used in agriculture with the aim of stimulant plant growth. Mode of action of biostimulant through different mechanisms with fertilizers, in which the mechanism of biostimulant activitate has so far been difficult to identify, due to the complexity of its bioactive compounds, so it must be proven through physiological and biological studies (Bulgari *et al*., 2015; Sharma *et al*., 2016). Biostimulant is not a nutrient or pesticide, but positively affected plant growth and health as well environmental friendly. Biostimulant contains bioactive compounds or microorganisms that can be applied to plants in order to improve the efficiency of nutrient absorption, tolerance of biotic, abiotic stress and can improve the quality of plants (Calvo *et al*., 2014; du Jardin, 2015; Halpern *et al*., 2015). Plant growth stimulate throughout the life cycle from seed germination, increased yield induction and plant quality including increased: plant metabolism, resistance mechanisms against abiotic and biotic stress (Kauffman *et al*. 2007; Keunen *et al.,* 2013; Kumari and Sairam, 2013; Wang *et al*., 2013; Wasternack and Hause, 2013; Zeng *et al.,* 2014; Calvo *et al.,* 2014), improved assimilation of nutrients and translocation, product quality, including sugar content , water efficiency used, and Soil fertility (Banks and Percival, 2012).

Bacteria are the most abundant microorganisms in the rhizosphere, most likely to affect plant physiology, especially through plant rooting colonization (Kloepper, 2007). Rhizospheric bacteria that colonized rooting play an important role in the regulation of plant growth triggers known as plant growth promoting rhizosphere (PGPR) (Kloepper *et al,* 1980). Various species of bacteria such as *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus* and *Serratia* are reported to increase plant growth (Kloepper *et al*., 1989; Glick, 1995).

The bacteria that colonized the rhizosphere can be identify and characterify according to their morphology and physiological influences on plants and the way bacteria interact with roots. A number of PGPR have been identified as important roles, especially their role as a promoting of plant growth (Kloepper *et al*, 1989; Glick, 1995; Kauffman *et al.* 2007; Keunen *et al.,* 2013; Kumari and Sairam, 2013; Wang *et al.,* 2013; Wasternack and Hause, 2013; Zeng *et al.,* 2014; Banks and Percival, 2012). The purpose of this research is to know the ability of *Bacillus spp* from root potato in producing biostimulant compound of plant growth.

2. Method

2.1 Source of Bacteria

The bacteria tested for the production ability of the biostimulant compound are: *Bacillus niabensis Strain* PT-32-1, *Bacillus niabensis, Bacillus subtilis Strain SWI16b, Bacillus subtilis Strain Strain HPC21, Bacillus cereus* Strain HY, *Bacillus moyavensis JCEN3, Bacillus moyavensis* UCMB 5075 and *Bacillus spp* derived from potato rizosphere (*Solanum tuberosum* L.) var. Hartapel. The bacteria were cultured on NA medium and then tested the potential for production of biostimulant compounds including the ability to solve phosphate, N fixation, production of ACC-deaminase, hormone, siderophore, and HCN.

2.2 Phosphate Solubilization Test

Phosphate solubilization was tested by using to the Pikovskaya method (Sundara and Shinha, 1962; Subba Rao, 1982). Measurement of solubilization phosphate concentration by using liquid Phikovkaya media. Absorbance was measured on a 693 nm UV-VIS spectrophotometer. Standard PO_4 curve (titrisol) is made from dilution with concentration with regression equation Y = 0.191 x + 0.048 where R2 = 0.957.

2.3 Free Nitrogen Fixation

The ability of bacterial isolates to bind free nitrogen was tested using Burk N-free medium (Stella and Suhaimi, 2010). Bacteria capable of growing on N-free Burk media are indicated as bacteria capable of fixing nitrogen.

2.4 Production of ACC-deaminase

Testing of the ability of ACC-deaminase enzyme-producing bacteria was performed by culturing bacterial isolates on Dworkin-Foster's minimum saline medium (DF) (Dworkin and Foster, 1958) enriched with 1-aminocyclopropane-1-carboxylate (ACC) as the only source of nitrogen (Glick, 1995). Bacterial capable of growing on this medium are indicated as ACC-deaminase-producing bacteria.

2.5 Production of Indole Acetic Acid (IAA)

The production of indole-3-acetic acid (IAA) auksin was tested using nutrient broth medium (NB) and Salkowski reagent (Gutierrez *et al*, 2009; Glickman and Dessaux, 1995). Pink color changes show IAA production. The concentration of auxin was measured using standard IAA curve with regression equation Y = 0.064 x + 0.09 where R2 = 0.995, made from serial dilution of IAA stock solution, absorbance was measured at 535 nm at UV-VIS spectrophotometer.

2.6 Production of Gibberellic Acid (GA3)

The production of gibberellic acid was tested according to standard methods (Borrow *at al,* 1995). The absorbent was measured at 254 nm on the UV-VIS spectrophotometer. GA3 concentration was measured using standard GA3 curve with regression equation Y = 0.888 x + 0.441 where R2 = 0.921, made from serial dilution of GA3 stock solution.

2.7 Siderophore Production Test

The production of bacterial isolate siderefors was tested using method described by Sivasakthivelan and Stella (2012). The bacterial isolates were incubated at 37ºC for 7 days. Absorbance was determined at 560 nm with sodium salicylate as the standard for salicylate estimation. Standard sodium salicylate is made from dilution with a concentration of salicylic nantrium with regression equation Y = 0.179 x + 0.027 where R2 = 0.902. Measurement of catechol type siderophores in absorbance of 700 nm with 2,3 dihydroxy benzoate acid (DHBA) as standard with regression equation Y = 0.209 x + 0.038 where R2 = 0.955.

2.8 HCN Test

Screening of bacterial isolates that produce hydrogen cyanide is carried out according to the method described by Bakker and Schippers (1987). Cyanide production was detected using a solution of cyanide detection solution. Changes in light brown to dark brown show the production of HCN.

2.9 Hypersenstivity Test

The bacterial suspension was injected 1 ml (106 CFU/ml with OD = 0.06 measured at 660 nm spectrophotometer absorption) in the tobacco leaf tobacco intervention area (Lelliot and Stead, 1987). In this test distilled water was used as a negative control and suspension of *Ralstonia solanacearum* bacteria as a positive control. The hypersensitivity reaction was observed after 12.24 and 72 hours of injection. Positive hypersensitive reaction response is shown in the presence of brown necrosis spots and dryness in leaf tissue or vice versa when shown negative hypersensitive reaction is not seen any changes in the leaf surface in the form of brown spots as a form

of necrosis.

3. Results and Discussion

The ability of *Bacillus spp* in the phosphate solubilization process, N fixation, results in ACC-deaminase, hormone production (IAA and GA3), production of siderophores, HCN and hypersensitivity reactions are presented in Table 1.

Table 1. *Bacillus spp* ability as Plant Growth Promoting Rhizobacteria

Bacteria	Phosphate solubilization (mg l^{-1})	Fixation of N	Production of ACC-deaminase	Production of Hormon (mg l^{-1})		Production of Siderophores		Production of HCN
				IAA	GA_3	Chatecol (mg l^{-1})	Salisilat (mg l^{-1})	
Bacillus niabensis Strain PT-32-1	14.237	+	+	5.114	2.866	2.257	3.228	+
Bacillus niabensis	-	+	+	3.173	5.140	0.685	3.055	+
Bacillus subtilis Strain SWI16b	12.492	+	+	5.816	6.389	1.709	3.347	+
Bacillus subtilis Strain HPC21	12.302	+	+	0.329	4.217	3.025	2.772	-
Bacillus cereus Strain HY	-	+	+	1.121	4.854	1.568	3.284	-
Bacillus moyavensis Strain JCEN3	13.210	+	+	0.393	5.784	4.206	4.121	-
Bacillus moyavensis Strain UCMB 5075	-	+	+	1.501	4.786	5.297	4.245	-
Bacillus spp.	-	-	-	0.574	5.880	4.206	4.121	-

Note: (+) positively produces ACC deaminase, N-fixation and HCN production (-) producing negative ACC deaminase, solubilization phosphate, N-fixation and production of HCN.

The ability of phosphate solubilization of bacteria was characterized by the formation of a halozone around bacteria on Phikovskaya media (Fig. 1a). The formation of clear zone is a process of degradation of tricalcium phosphate in Phikovskaya media as a form of bacterial ability to dissolve phosphate. From eight bacterial strains tested there were only four bacterial isolates showing the ability to dissolve phosphates: *Bacillus niabensis* Strain PT-32-1, *Bacillus subtilis* Strain SWI16b, *Bacillus mojavensis* Strain JCEN3 and *Bacillus subtilis Strain* HPC21 with different levels of phosphate dissolution (Table 1).

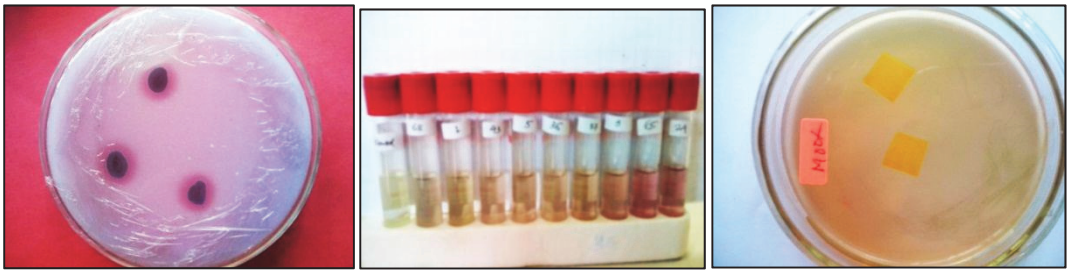
The ability of bacteria growing on Dworkin-Foster selective medium are indicated as bacteria that have the ability to use ammonium sulfate as a bacterial constitutive ability to produce ACC-deaminase. Selected bacteria in Dworkin-Foster medium are able to use ACC substrate as the only nitrogen source and have the ability to produce ACC-deaminase. The ACC-deaminase enzyme is present in many rhizosphere bacteria, where bacteria take ACC secreted by the roots and convert it to α-ketobutyrate and ammonia (Glick *et al*., 2007). In Table 1 showed that bacteria showing ACC-deaminase activity also indicate N fixation activity. This showed that there is a relationship between N fixation activity and ACC-deaminase activity. Based on several studies of bacteria capable of growing on Burk N-free media indicated that bacteria capable of fixing nitrogen (Ding *at al,* 2005; Döbereiner and Pedrosa, 1987; Döbereiner *et al.* 1995, Mantilla-Paredes *et al*. 2009, Sgroy et al, 2009 Silva dan Melloni, 2011) can form the pellicle (Lea-Madi *et al*. 1988; da Silva *et al*., 2013), can produce ammonium (Shanmugam and Valentine, 1975; Saribay, 2003) and have nitrogenase activity (da Silva *et al*, 2013; Dilworth, 1966; Ueda *et al*. 1995; Ohkuma et al. 1996; Widmer *et al.* 1999; Kirshtein *et al.* 1991; Garbeva *et al.* 2001; Sessitsch *et al*. 2005; Reiter *et al*. ; Chelius and Triplett, 2001).

The ability to produce IAA varies from each strain of *Bacillus* (Table 1). The difference in IAA concentrations produced by the various bacteria tested was due to differences in bacterial capabilities utilizing tryptophan. The concentration of IAA produced by bacteria depends on the substrate of L-tryptophan in the medium, which is detected by pink change after the addition of Salkowski reagent in the test process (Figure 1b).

All of the tested *Bacillus* strains have the ability to produce gibberellic acid (GA3). Levels of concentration

gibberellic acid are presented in Table 1. It is estimated that bacteria can increase GA levels in bacterial cultures due to GAs production, deconjugating GAs from root exudates or inactive GA hydroxylating (Bottini *et al*., 2004).

The number of bacterial isolates capable of secreting siderophores were the same as the number of bacterial isolates capable of producing ACC-deaminase, IAA, GA and N fixing in all tested *Bacillus* strains. Siderophore microbials can be biocontrol for plants because of their competitive effects with pathogens (Hofte, 1993). Production of siderophores with maximum or minimum concentration is not found in the same strain of bacteria but in different bacterial strains. These findings were showed in *Pseudomonas fluorescens,* where the maximum and minimum siderophore concentrations produced found on the same bacterial isolate (Saranraj, *et al*., 2013; Sivasakthivelan and Stella 2012.) The difference was predicted cause by differences in synthesis capability of both types Siderophore from bacterial isolate *Bacillus spp.* Based on the results of the study, *Bacillus niabensis* Strain PT-32-1, *Bacillus niabensis* and *Bacillus subtilis* Strain SWI16b positively produce hydrogen cyanide characterized by color change on cyanide detection solution indicator paper to brown (Figure 1c). Hydrogen cyanide produced by many rhizobacteria has been postulated to play a role in the biological control of pathogens (Defago *et al*., 1990), and is said to be an inducer of plant resistance and growth trigger (Schippers, 1993).



(a) (b) (c)

Figure 1. (a) Hallozone phosphate on solubilization test, (b) Color change of Bacteria suspension on IAA test, (c) result of HCN test

Hypersensitivity test results showed that all bacteria did not trigger hypersensitive reactions in tobacco leaves (Table 2). Suspension of bacteria injected in tobacco plants was not pathogenic to plants. This reaction was only found in bacterial isolates *Ralstonia sp*. as a positive control. The

neocrotic symphtoms was only shown by *Ralstonia sp* treatment as a positive control. Klement *et al*., (1990) suggests that at this stage there was a reaction between phenol compounds contained in the vacuole with subsation present in the cytoplasm and formed cytololic compound resulting necrosis symptoms.

Table 2. Hypersensitivity Response Bacterial Isolates in Tobacco Leaves

Bacteria	Hipersensitive Reactions			
	12 hours	24 hours	48 hours	72 hours
Bacillus niabensis Strain PT-32-1	-	-	-	-
Bacillus niabensis	-	-	-	-
Bacillus subtilis Strain SWI16b	-	-	-	-
Bacillus subtilis Strain HPC21	-	-	-	-
Bacillus cereus Strain HY	-	-	-	-
Bacillus mojavensis Strain JCEN3	-	-	-	-
Bacillus moyavensis UCMB 5075	-	-	-	-
Bacillus spp	-	-	-	-
Akuades steril	-	-	-	-
Ralstonia solanasearum	+	+	+	+

Note: + positive shows hypersensitivity reaction, no hypersensitivity reactions occur.

4. Conclusion

Bacillus niabensis Strains of PT-32-1, *Bacillus subtilis* Strain SWI16b, *Bacillus mojavensis* Strain JCEN3,

Bacillus subtilis Strain HPC21, and *Bacillus moyavensis* UCMB 5075 have potential as biostimulant active ingredients of plant growth because physiological characters capable of solubilization phosphate, nitrogen fixition, producing ACC-deaminase, IAA, GA, and siderophores as well as non hypersensitive.

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