Journal of Sugar Beet
http://jsb.areeo.ac.ir/
Journal of Sugar Beet 2015, 31(1)

Detection of hydrogen cyanide biosynthetic gene in Pseudomonas fluorescens as a control agent of Rhizoctonia solani growth

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Konjedi M, Vatandoost J, Janatabadi A. Detection of hydrogen cyanide biosynthetic gene in *Pseudomonas fluorescens* as a control agent of *Rhizoctonia solani* growth. J. Sugar Beet. 2015; 31(1): 31-36.

Received April 26, 2014; Accepted April 28, 2015

ABSTRACT

In order to detect hydrogen cyanide gene and to determine *Pseudomonas fluorescens* efficiency in producing hydrogen cyanide and controlling the growth of *Rhizoctonia solani* pathogen (the causal agent of sugar beet root and crown rot), samples were collected from sugar beet rhizosphere fields in Sabzevar, Iran. After soil sampling and purification, 31 *Pseudomonas spp.* isolates were separated on specific Pseudomonas agar F medium, and *Pseudomonas fluorescens* strains were identified and isolated based on microbial tests. PCR results for the detection of hydrogen cyanide biosynthesis gene showed that three strains contained hydrogen cyanide biosynthetic gene. The qualitative evaluation of hydrogen cyanide production in microbial medium showed that all the strains were capable of producing different amounts of hydrogen cyanide. To determine the growth inhibition rate of *Rhizoctonia solani* by *Pseudomonas fluorescens*, the percentage of fungus growth in the presence of bacteria was calculated. Among the three *Pseudomonas fluorescens* strains, C7 showed the greatest inhibition rate and hydrogen cyanide production rate, so it can be recommended as a suitable candidate for the pathogen biocontrol.

Keywords: Rhizoctonia root and crown rot, sugar beet, *Pseudomonas fluorescens*, biocontrol

INTRODUCTION

Sugar beet is an important industrial crop which is a source of one of the basic needs of the people named sugar. One of the main diseases of sugar beet is root rot (Hecker and Ruppel 1977). Root rot usually occurs in fields where sugar beet roots are exposed to excessive soil moisture (Habibi 1975). The cause of root and crown rots, dry rot, and violet root rot in sugar beet is a fungus from the rhizoctonia family including a soil-borne species called *rhizoctonia solani*. The fungus attacks sugar beet root and creates purplish brown networks of intertwined threads on the root surface. Sometimes, these threads cover the entire surface of the root, blocking phloem and hindering the vital activities of the plant (Sheikholeslam et al. 2006). Since this species is responsible for root and crown rot, seedling death, and leaf blight of sugar beet, enormous genetic diversity has been reported in its population which has been titled as complex species (Vilgalys and Cubeta 1994). In Iran, *R. solani* was first reported on tomato plants and pines by Sharif and Ershad and on bean plants by Manouchehri and Qanadzadeh (Balali and Moharabi 2006, Ershad 1977). In addition to *R. solani*, a lot of other pathogenic fungi are responsible for sugar beet root rot at different phases (Whitney and Duffos 1991). *Pythium aphanidermatum (Edson) Fitzp*. is known as a causal agent of seed rot, seedling death, and root rot in Iran (Ahmadinejad and Okhovat 1976). Also, *Rhizopus arrhizus Fischer* has been identified as a cause of crown rot of sugar beet in Iran (Habibi 1977).

An approach to control soil-borne pathogens is their chemical control which not only contami-

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nates the environment but also affects natural microflora of the soils adversely and impairs their fertility. For this reason, the biological control gains an increasing importance (Adesina et al. 2007) and is recommended to avoid the negative impacts of agrochemical pesticides on the environments and consumers (Arcury et al. 2003). These negative impacts include biodiversity loss of soil microorganisms, the detrimental effects of pesticide runoffs on marine systems and the development of fungicide resistance in pathogens (Gerhardson 2002), acute health problems rising from farmers' exposure to chemical herbicides (Arcury et al. 2003), the residuals of pesticides on most food items including fruits and vegetables that endangers consumer health, and the increase in pesticide costs, especially in low-income countries.

The beneficial role in plant growth enhancement of soil-borne bacteria, among which Pseudomonas is of extreme importance, has been known for over a century. Evidence shows that the application of Pseudomonas to seeds contributes to the protection of the seeds and plants against soil-borne pathogens and increase in their yield (Behbodi and Sharifi 2006). Most isolates of soil-borne Pseudomonas stimulate root growth and colonization and thereby improve plant growth by inhibiting the growth of pathogens through producing such compounds as antibiotics, siderophore, hydrogen cyanide, and protease or directly by generating plant hormones. In this regard, the application of biocontrol Pseudomonas in agriculture to enhance soil fertility has gained a high importance in the last 30 years (Weller 2007).

Certain strains of *Pseudomonas fluorescens* inhabiting in the rhizosphere have attracted much attention in recent years due to their high capability in plant protection against fungal, bacterial and nematode pathogens (Haas and Défago 2005). *P. fluorescens* has an inhibitory effect on a wide range of plant pathogens and operates through diverse inhibitory mechanisms (Kumar and Bude 1992, Tian and Riggs 2000). In addition to its capability in propagating and inducing systemic resistance, the production of volatile metabolites, such as hydrogen cyanide by the soil-borne bacteria, it is the most effective and efficient mechanism for the biological control of plant diseases, but it has not been considered extensively. Hydrogen cyanide effectively blocks the pathway of cytochrome oxidase and is very toxic to all aerobic microorganisms at picomolar concentrations (Jayaprakashvel and Mathivanan 2011, Pal and Gardener 2006). Cyanide binds to ferric iron (Fe^{3+}) of the enzyme cytochrome oxidase of the respiration chain and hinders the aerobic respiration of prokaryotic and eukaryotic cells. The production of hydrogen cyanide by some *P. fluorescens* contributes to the suppression of plant diseases. Furthermore, Pseudomonas can produce antibiotic and/or hydrogen cyanide and thereby compete with pathogens on nutrients or it can compete with pathogens by inducing systemic acquired resistance in plants after root colonization. Pseudomonas needs adequate iron to produce hydrogen cyanide.

By producing antibiotic, siderophore, and hydrogen cyanide, *P. fluorescens* strain CHAO plays a role in the suppression of black and white rot disease of tobacco in which it seems that hydrogen cyanide production is more influential in the suppression of this disease and is of more specific importance (Pal and Gardener 2006). In a study on 10 strains of *Pseudomonas fluorescens* isolated from the soils of maize, rice, and alfalfa fields, it was observed that the isolates could be used as potential biological fertilizers and as biocontrol agents (Suresh et al. 2010). Another strain of Pseudomonas, *Pseudomonas pathogenesis Pf-5* used to be named as a component of *Pseudomonas fluorescens*, inhabits the surface of seeds and roots of the plants and can protect the plant against fungal infections and pathogenic bacteria (Kidarsa et al. 2013, Ramette et al. 2011). Saharan and Nehra (2011) reported that when fields were surrounded with Pseudomonas, the grain yield was increased due to secondary metabolite production. Rhizobacteria has inhibitory effects as well as impacts akin to those of Pseudomonas so that they can play a critical role in the biological control of fungal pathogens that are transferred through the soil (Kapsalis et al. 2008). In some reports, the microbial production of hydrogen cyanide as an antifungal medication has been regarded as an important trait for the control of root-infecting fungi (Adhikari et al. 2013, Ramette et al. 2003) so that crown rot of sunflowers was suppressed by *Pseudomonas fluorescens* through the production of hydrogen cyanide (Adhikari et al. 2013, Shivani et al. 2005). It has been documented that at least 40% of Pseudomonas isolated from potato rhizosphere produced hydrogen cyanide in vitro. Suresh et al. (2010) also reported the production of hydrogen cyanide by *Pseudomonas fluorescens* at different rates.

Given the economic importance of sugar beet, the high pathogenic potential of Rhizoctonia spe-

cies in different stages of sugar beet growth, and the crucial role of hydrogen cyanide produced by bacteria in mitigating the severity of sugar beet crown and root rot, the present study was aimed to identify *Pseudomonas fluorescens* in the rhizosphere of sugar beet fields in Joveyn and Khoshab of Sabzevar, Iran in order to detect the gene responsible for the biosynthesis of hydrogen cyanide by this bacteria and to explore the inhibitory effect of the isolates on the fungus *R. solani* under *in vitro* conditions. The results can contribute to taking effective decision for the management of this pathogen.

MATERIALS AND METHODS

Sampling and identification of Pseudomonas fluorescens

Samples were collected from five fields in Bahr Abad of Joveyn and Khoshab that were infested to *R. solani*. Ten samples were taken from each field in a zigzag pattern using auger. Samples of each zigzag pattern were mixed to one another to reach the total microorganisms. Fields were labeled with A, B, C, D, and E letters and samples were coded.

For bacteria culture, first, a suspension of 10 g of sample mix and 100 mL of distilled water was prepared and was left for 2 hours until the supernatant became clear. Then, the linear culture was done from each sample on Pseudomonas agar F medium. After incubation at 28°C for 48 hours, the grown colonies were examined in terms of the appearance of colonies, colony diameter and halo, and their color and size. Next, colonies were grown again on Pseudomonas agar F medium for their purification and they were re-cultured until a single colony remained.

To identify the bacteria, the purified colonies in each Petri dish were subjected to gram staining test, catalase test, oxidase test, aerobic-anaerobic test, gelatin melting, citrate test, nitrate reduction, MRVP test, resistance to kanamycin, and H_2S production in the selected isolates. All these 10 identification tests were conducted by foliar method (Holt et al. 1994).

Detection of the gene hcnBC

DNA was extracted from the identified *Pseudomonas fluorescens* bacterial by boiling with KOH 5% for which first 500 µl of bacteria suspension with 25 µl of KOH 0.5 M was boiled in water for 2 min. Then, another 25 µl of KOH was added and it was boiled for another 2 min. Finally, the infiltrated suspension was centrifuged at 9000 rpm for 4 min. The supernatant contained the DNA of the bacteria. It was stored at -20°C.

Table 1. PCR primers applied to amplify the biosynthesis genes of *hcn*BC

Primer	Primer name	Nucleotide sequence
Forward primer	*hcn*-F	ACT GCC AGG GGC GGA TGT GC
Reverse primer	*hcn*-R	ACG ATG TGC TCG GCG TAC

The extracted DNAs were subjected to a PCR test with designed primers *hcn*-F and *hcn*-R (Table 1). Primers were designed by GeneRunner software. PCR was conducted on 25 µl of reaction mixture containing 20 ng of DNA, 2.5 µl of 10 X PCR buffer, 1.5 µl of 50 mM $MgCl_2$, 1 µl of 10 mM dNTP, 1 µl of 0.2 mM of each primer, and 0.5 units of the enzyme Taq polymerase under the following conditions: initial denaturation at 94°C for 4 min followed with the chain including 60 seconds at 94°C, 60 seconds at 52°C and 2 min at 72°C. Then, samples were subjected to 72°C for five min to ensure the completion and development of the primers. The procedure and concentration of the materials were similar for all samples. The products of PCR were detected by electrophoresis on Agar gel 1.55 using TAE 1X buffer. The gel was colored with ethidium bromide and was examined in electrophoresis tank. The consecutive replications and changes in conditions (thermal gradient, DNA gradient, $MgCl_2$ gradient) were all aimed to extract bands from samples.

Hydrogen cyanide production in isolates

The rate of hydrogen cyanide production by each sample of *Pseudomonas fluorescens* was determined by Alström and Burns (1989)'s procedure. First, a filter paper was placed on the door of the Petri dish, and then, they were sterilized. Nutrient agar (NA) culture medium was prepared with 4.4 g/l of glycine and after cooling, it was poured into sterile Petri dish. After 24 h, the isolates were cultured in a linear pattern. The filter paper was wetted with 2 ml of sterile picric acid and was placed on the medium. The Petri dishes were capped, and the reaction with picric acid was allowed on the culture medium in order for antagonist rhizobacteria to produce gaseous metabolites. After incubation at 25±2°C for one week, the color change of the filter paper was evaluated as a potential indicator of hydrogen cyanide generation by antagonist rhizobacteria.

Table 2. Biochemical and physiological traits of three strains of *Pseudomonas fluorescens* among 31 isolates strains of Pseudomonas

Strain	Gram	Oxidase	Catalase	Citrate	Nitrate	Gelatin melting	Movement	H_2S production	Indole	MIR	VP	Resistance to kanamycin	Shape	Aerobic growth	Anaerobic growth
C3	-	+	+	+	+	+	+	-	-	+	+	Susceptible	Bacillus	+	-
C5	-	+	+	+	+	+	+	-	-	+	-	Susceptible	Bacillus	+	-
C7	-	+	+	+	+	+	+	-	-	+	-	Susceptible	Bacillus	+	-

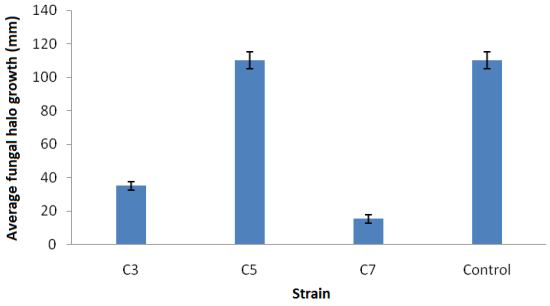

Figure 1. The extent of the growth of fungal halo against strains

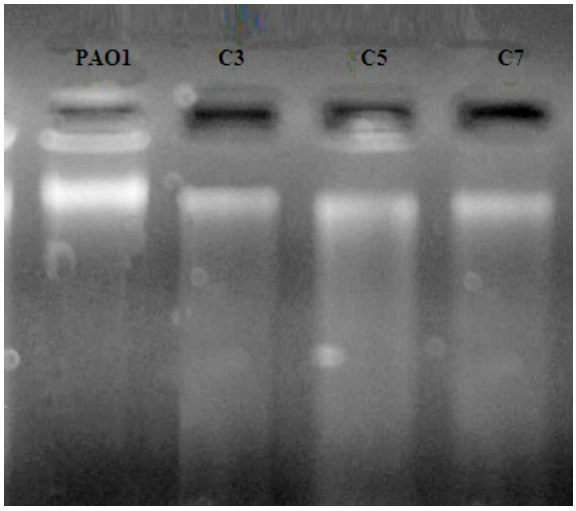

Figure 2. Results of DNA extraction

Inhibition of pathogen growth

To isolate *R. solani* fungi, first 1×1×1 cm pieces were detached from healthy and infected sugar beets. After sterilizing, samples were separately placed in the middle of Petri dish containing PDA culture medium and were incubated at 25°C for one week. Fungi were detected by Parmeter Jr. and Whitney (1970)'s method. To figure out the optimal growth temperature, the fungi were grown at different temperatures.

To estimate the inhibitory effect of *Pseudomonas fluorescens* isolates on the growth of *R. solani*, we used the dual culture of the bacteria and fungi in PDA culture medium. First, the bacteria were cultured at the whole surface of the Petri dish. Twenty-four hours after bacteria culture at 25°C, a 0.5 × 0.5 cm disc from the margin of the three-day culture of pathogenic fungus was placed inversely in the middle of Petri dish in which bacteria had grown. After the incubation of the Petri dish at 25°C for 48 h, the growth of fungi in dual culture and in the presence of the bacteria was examined.

The study was conducted in a randomized complete block design with six replications and the growth rate of fungal mycelium was measured in the presence of the bacteria. Data were statistically analyzed using SAS software. Treatments were grouped by Duncan's multiple range Test ($P \geq 1\%$).

RESULTS

Pseudomonas fluorescens isolation and identification

Identification keys were employed and 31 strains of Pseudomonas bacterium were isolated among which three strains were identified as *Pseudomonas fluorescens*. These three strains were identified from field C and they were coded as C7, C5, and C3. Results of identification tests are presented in Table 2.

Inhibitory effect between bacterium and fungus

The growth rate of *R. solani* in the presence of PS isolates was estimated by dual culture test. According to this test, sample C5 exhibited the highest rate of fungal halo growth around bacterium and sample C7 showed the lowest one (Figure 1). This means that the strains C7, C5, and C3 had the highest, intermediate, and no inhibitory effects, respectively.

DNA extraction

After DNA extraction by KOH5%, the quality of the extracted DNA was examined on electrophoresis 1% to ensure their correct extraction (Figure 2).

PCR test to detect the gene hcnBC

Using the PCR method and *hcn*-F and *hcn*-R primers, the DNA fragment with the approximate length of 587 base pair (bp) was amplified from the gene group of biosynthesis of the hydrogen cyanide secondary metabolite. The expected length of the product between these primers is 587 base including 156 terminal base from the gene *hcn*B and 436 initial base from the gene *hcn*C. According to the results of this phase and their comparison with the standard strain PAO1, the strains C7, C5 and C3 were found to have hydrogen cyanide biosynthesis gene (Figure 2).

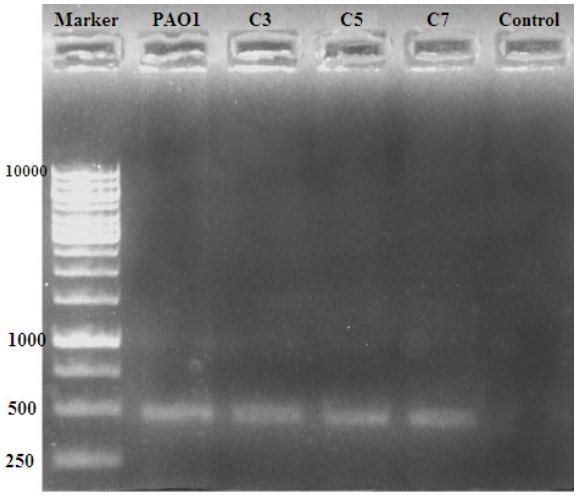

Figure 3. Band pattern derived from PCR

Hydrogen cyanide production by antagonistic isolates

The qualitative examination of hydrogen cyanide production by Alström method showed that all strains were capable of producing hydrogen cyanide but at different rates. The rate of hydrogen cyanide production is assessed with the intensity of the color. After samples incubation at 25±2°C for one week, the color change of filter paper to brown was a sign of weak production of hydrogen cyanide in antagonist rhizobacteria. If the filter paper turns to orange-brown, hydrogen cyanide is produced at a moderate rate, and color change to red-orange is a sign of its strong production rate. No color change implies no hydrogen cyanide production, but it was not observed in any of our samples. These observations showed that the highest rate of the gas production was related to the sample C7 after the control sample PAO1 and the lowest rate was related to the sample C5.

DISCUSSION

A lot of pathogenic fungi cause root rot in sugar beet at different stages among which Rhizoctonia is responsible for the crown and root rot, dry rot, and violet rot of roots at different growth stage (Whitney and Duffos 1991). Thus, it is of crucial importance to explore the methods for the biological control of these diseases. Since the role of *Pseudomonas fluorescens* has been established in the control of fungal in various plant species, the identification of *Pseudomonas fluorescens* in the rhizosphere of sugar beet fields and examination of their potential to suppress *R. solani* growth can contribute to the selection of soil biocontrolling bacteria. According to the observations of fungus halo growth in dual culture of bacteria and fungi, it was revealed that the sample C7 had the highest inhibitory effect against *R. solani*. This inhibition potential can be related to the production of secondary metabolites including hydrogen cyanide, siderophore, and phenazine.

Given the role of hydrogen cyanide produced by bacteria in mitigating the diseases of the crown and root rots, the presence of the gene for hydrogen cyanide and its production by *Pseudomonas fluorescens* bacteria were studied too. According to the results, all three strains C3, C5 and C7 had the gene for hydrogen cyanide biosynthesis. The qualitative assessment of hydrogen cyanide production showed that although all strains could produce this gas, its production rate is different among them. Results showed that the strain C7 exhibited the highest rate of hydrogen cyanide production after control, which is consistent with its inhibitory power. Thus, it seems that hydrogen cyanide production is among the main factors responsible for the inhibition of the growth of the studied fungus, so this isolate can be used as a potential biological fertilizer and a biocontrol factor. Hydrogen cyanide effectively blocks the cytochrome oxidase pathway and is very toxic to all aerobic microorganisms at picomolar concentration.

These results are in line with other studies on the fungal growth inhibitory effect of *Pseudomonas fluorescens* (Pal and Gardener 2006, Girard et al. 2006) and demonstrate that hydrogen cyanide production by some *Pseudomonas fluorescens* species is involved in the suppression of plant disease. *Pseudomonas fluorescens* strain CHAO suppresses the black and white rot of tobacco by producing antibiotic, siderophore and hydrogen cyanide. It seems that hydrogen cyanide has the leading role in disease suppression (Pal and Gardener 2006). Another study has shown that hy-

drogen cyanide production by *Pseudomonas fluorescens* protects tobacco against black root rot (Girard et al. 2006). So, it can be concluded that the production of volatile metabolites like hydrogen cyanide by soil bio-controlling bacterial can be one of the most effective and efficient mechanisms to protect plants against soil-borne pathogens, but their role has been underestimated. These results can be supplemented by further studies on the feasibility of using these bacteria as biofertilizer.

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