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Viability of Antifungal Metabolite Producing *Pseudomonas* Bacteria

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Abstract: The objectives of this study were to determine the suitability of transport medium (ice jells) and estimate the duration of viability of *Pseudomonas* in the transport medium. Bacteria of the genus *Pseudomonas* comprise a large group of the active biocontrol strains as a result of their general ability to produce a diverse array of potent antifungal metabolites. These include simple metabolites such as 2,4-diacetylphloroglucinol, phenazine-1-carboxylic acid and pyrrolnitrin [3-chloro-4-(2-nitro-3-chlorophenyl)-pyrrole], as well as the complex macrocyclic lactone, 2, 3-de-epoxy-2, 3-didehydro-rhizoxin. Pyrrolnitrin is active against *Rhizoctonia* sp., *Fusarium* sp. and other pathogenic fungi and it has been used as a lead structure in the development of a new phenylpyrrole fungicide. The survival rates of four different pseudomonad strains after continuous incubation for 4 h in the cold temperature (4°C) were: 94.8% for *P. putida* strain CBD, 94.5% for *P. aeruginosa* No. BRCH and 62.1% for *Pseudomomas* species *(fluorescent)* with lowest survival rate of 33.5% for *P. aeruginosa* strain H. Since, there were no drastic reductions in the survival rates, the study findings suggest that the transport medium would be generally suitable for these cold-sensitive bacteria.

Key words: Bacteria, antifungal metabolite, pyrrolnitrin, pathogenic fungi, reduction, survival rate, transport medium

INTRODUCTION

Pseudomonas is a chemoorganotrophic Gram negative bacterium, that is known to cause bovine mastitis (Buxton and Fraser, 1977). The fluorescent pseudomonads are of particular interest because a number of these species are plant pathogens (Ligon *et al*., 1999) and at least one (*P. aeruginosa*) is a human pathogen (Howell and Stipanovic, 1980). It is now well established that some bacteria have the ability to produce antifungal compounds involving both the metabolites and enzymes. The biological mechanism underlying this phenomenon is known as bio-control and the bacteria of the genus *Pseudomonas* comprise a large group of active bio-control strains as a result of their general ability to produce a diverse array of potent antifungal metabolites. These include simple metabolites such as 2, 4-diacetylphloroglucinol, phenazine-1-carboxylic acid and pyrrolnitrin [3-chloro-4-(2-nitro-3-chlorophenyl)-pyrrole], as well as the complex macrocyclic lactone, 2, 3-de-epoxy-2, 3-didehydro-rhizoxin. The study of biochemistry and the mechanism of formation of these metabolites has proved useful in several ways. Pyrrolnitrin is active against *Rhizoctonia* sp., *Fusarium* sp., *Pythium ultimum*, *Gaeumannomyces graminis* var. *trici* and

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Thielaviopsis basicola and other pathogenic fungi and has been used as a lead structure in the development of a new phenylpyrrole fungicide (Weller and Cook, 1983; Ayers, 1960; Nickerson and Sinskey, 1973; Walker, 1975; Mirleau *et al*., 2005). In addition, pyrrolnitrin has been used for years as a model for the study of mechanisms involved in the chlorination of organic molecules. The *Pseudomonas* sp. have been investigated as potential biological control agents due to their ability to produce a battery antifungal compounds against a range of agronomically important fungal diseases (Misaghi and Grogan, 1969; Curtin *et al*., 1961; Weller and Cook, 1983; Walker, 1975). The fluorescent pseudomonads have also been considered as biological control agents against various root diseases (and in the degradation of oil spills (McGill *et al*., 1981; Ayers, 1960; Mirleau *et al*., 2005). The fluorescent *pseudomonads* have been frequently reported as contaminants in certain meat and dairy products, in the biodegradation of pesticides and chemical wastes and in the industrial fermentation of organic acids and have been suggested as indicators of water quality (Krieg and Holt, 1984; Howell and Stipanovic, 1979; Weller and Cook, 1983; McGill *et al*., 1981). Many *Pseudomonas* strains are not easily maintained at refrigerated temperature and relatively die within a short time at 4°C. Because many *Pseudomonas* strains are inactivated at low temperatures and are cold sensitive (Krieg and Holt, 1984; Wolf, 1972).

Haas *et al*. (2000) reported that root diseases caused by fungal pathogens can be suppressed by certain rhizobacteria that effectively colonize the roots and produce extracellular antifungal compounds. To be effective, biocontrol bacteria need to be present at sufficiently high cell densities. These conditions favor the operation of positive feedback mechanisms that control the production of antifungal compounds in biocontrol strains of fluorescent pseudomonads, via both transcriptional and post-transcriptional mechanisms. Landa *et al*. (2002) stated that the indigenous populations of 2, 4-diacetylphloroglucinol (2, 4-DAPG)-producing fluorescent *Pseudomonas* sp. that occur naturally in suppressive soils are an enormous resource for improving biological control of plant diseases. Population densities of strains belonging to genotypes D and P were significantly greater than the densities of other genotypes and remained above log 6.0 CFU (g of root)$^{-1}$ over the entire 15 week experiment. Genetic profiles generated by rep-PCR or restriction fragment length polymorphism analysis of the 2, 4-DAPG biosynthetic gene *phlD* were predictive of the rhizosphere competence of the introduced 2, 4-DAPG-producing strains.

Johansson and Wright (2003) reported that the influence of environmental factors during isolation on the composition of potential biocontrol isolates is largely unknown. Bacterial isolates that efficiently suppressed wheat seedling blight caused by *Fusarium culmorum* were found by isolating psychrotrophic, root-associated bacteria and by screening them in a bioassay that mimicked field conditions. Isolates in this group were identified as *Pseudomonas* sp., they were fluorescent on King's medium B and had characteristic crystalline structures in their colonies. Members of this morphological group grow at 1.5°C and produce an antifungal polyketide (2, 3-deepoxy-2, 3-didehydrorhizoxin [DDR]). In summary, this study described some isolation factors that are important for obtaining disease-suppressive bacteria in our system and is described a novel group of biocontrol pseudomonads.

Bergsma-Vlami *et al*. (2005) found that the genotypic diversity of antibiotic-producing *Pseudomonas* sp. provides an enormous resource for identifying strains that are highly rhizosphere competent and superior for biological control of plant diseases. They developed a simple and rapid method to determine the presence and genotypic diversity of 2,4-diacetylphloroglucinol (DAPG)-producing *Pseudomonas* strains in rhizosphere samples. Denaturing Gradient Gel Electrophoresis (DGGE) of 350 bp fragments of *phlD*, a key gene

involved in DAPG biosynthesis, allowed discrimination between genotypically different *phlD*$^+$ reference strains and indigenous isolates. Collectively, these results demonstrated that DGGE analysis of the *phlD* gene allows identification of new genotypic groups of specific antibiotic-producing *Pseudomonas* with different abilities to colonize the rhizosphere of sugar beet seedlings.

The objectives of this study were to determine the suitability of transport medium (ice jells) and estimate the duration of viability of *Pseudomonas* in the transport medium.

MATERIALS AND METHODS

Four *Pseudomonas* strains such as *Pseudomonas aeruginosa* strain BRCH, *Pseudomonas* aeruginosa strain H, *Pseudomonas putida* strain BCD and *Pseudomonas* sp. (fluorescent) were obtained from American Type Culture Collection (ATCC). Each strain was grown in nutrient broth (Difco) in 125 mL Erlenmeyer flask and incubated in a gyratory water bath shaker at room temperature (23°C). After 48 h, 1 mL of the incubated culture was transferred to another set of tubes containing 5 mL of skim milk broth (Difco). At time zero, a series of dilutions ranging from 10^{-1} to 10^{-7} were done by pipetting 1 mL of the skim milk culture mixture into 9 min nutrient broth (dilutent). Spread plating was performed in duplicate by pipetting 0.1 mL of each of the final dilutent on Proteose Glycerol Agar (PPG) plates (Difco). However, delayed pipetting was avoided to eliminate the cell's attachment to the pipettes. The above procedures were repeated after each one hour (up to 4 h) incubation in a small ice chest containing 3 jell ice bags and PPG plates were incubated up to 24 h at room temperature (23°C). The standard plate counts (Krieg and Holt, 1984) were used to monitor viable cell counts of four different *Pseudomonas* strains. Only those plates with 30-300 colonies were selected. The valid counts were multiplied by the dilution factor of each plate to determine the total plate counts which represented the original number of bacteria per milliliter of milk. The survival percent of a strain was computed by dividing the number of viable cells after incubation for 4 h by the number of viable cells at zero time incubation. This experiment was designed to assure that if *Pseudomonas* is isolated, then the transportation medium, temperature and time to laboratory would not affect isolation negatively.

RESULTS AND DISCUSSION

The results showed that the viability of four different *Pseudomonas* strains was considerably affected by the incubation time (Table 1). The *Pseudomonas aeruginosa* strain BRCH count declined gradually from 9.1×10^8 at zero hour to 8.9×10^8 at 3 h and 8.6×10^8 at 4 h of incubation. The *P. aeruginosa* strain H showed marked decline in the colony count from 2×10^9 to 6.7×10^8 at the end of continuous incubation for 4 h. The *P. putida* strain CBD showed gradual decrease in the colony count from 5.8×10^8 to 5.6×10^8 at 2 h and further decreased to 5.5×10^8 after continuous incubation for 4 h. The *Pseudomonas* sp. (fluorescent) showed moderate decline in the colony count from 6.6×10^8 at 0 h, to $6.5\times10^\circ$, $5.8\times10^\circ$, 4.7×10^8 and 4.1×10^8 at 1, 2, 3 and 4 h incubation period, respectively. However, survival rates of four different pseudomonad strains after continuous incubation for 4 h in the cold temperature (4°C) were 94.8% for *P. putida* strain CBD (the highest survival rate), 94.5% for *P. aeruginosa* No. BRCH and 62.1% for Pseudomonas species (fluorescent). On the other hand, the lowest survival rate of 33.5% was for *P. aeruginosa*

Table 1: Viable cell count of four different strains of *Pseudomonas* sp. at dilution 10^{-6} of skim milk[1]

	Bacterial species							
	R. aeruginosa No. BRCH ATCC 19429		*P. aeruginosa* No. H ATCC 19429		*R. putida* No. CBD ATCC 17522		*Pseudomonas* sp. (*fluorescent*) ATCC 17518	
Time (h)	Colony per plate	No. of viable (Cells mL^{-1})	Colony per plate	No. of viable (Cells mL^{-1})	Colony per plate	No. of viable (Cells mL^{-1})	Colony per plate	No. of viable (Cells mL^{-1})
0	91	9.1×10^{8}	200	2.0×10^{9}	55	5.8×10^{8}	66	6.6×10^{8}
1	91	9.1×10^{8}	180	1.8×10^{9}	58	5.8×10^{8}	65	6.5×10^{8}
2	89	8.9×10^{8}	103	1.03×10^{9}	56	5.6×10^{8}	58	5.8×10^{8}
3	89	8.9×10^{8}	68	6.8×10^{9}	57	5.7×10^{8}	47	4.7×10^{8}
4	89	8.9×10^{8}	67	6.7×10^{9}	55	5.5×10^{8}	41	4.1×10^{8}
Survival (%)	94.5		33.50		94.8		62.12	

[1]Plating factor =10

strain H. Since there were no drastic reductions in the survival rate of different strains which suggests that the transport method would be suitable even for these cold-sensitive bacteria. The experimental findings were comparable to those reported by Johansson *et al.* (2003), who reported that the influence of environmental factors during isolation on the composition of potential biocontrol isolates is largely unknown. They identified Isolates in this group as *Pseudomonas* sp., they were fluorescent on King's medium B and had characteristic crystalline structures in their colonies. Members of this morphological group grow at 1.5°C and produce an antifungal polyketide (2, 3-deepoxy-2, 3-didehydrorhizoxin [DDR]).

However, the study results do not agree with those of Krieg and Holt (1984) and Wolf (1972) who stated that many *Pseudomonas* strains are not easily maintained at refrigerated temperature and relatively die within a short time at 4°C. Because many *Pseudomonas* strains are inactivated at low temperatures and are cold sensitive.

CONCLUSIONS

The viability of four different *Pseudomonas* strains was considerably affected by the incubation time. The survival rates of four different pseudomonad strains after continuous incubation for 4 h in the cold temperature (4°C) were 94.8% for *P. putida* strain CBD (the highest survival rate), 94.5% for *P. aeruginosa* No. BRCH and 62.1% for *Pseudomonas* species (*fluorescent*). However, the lowest survival rate of 33.5% was obtained for *P. aeruginosa* strain H. Since, there were no drastic reductions in the survival rate of different strains which suggests that the transport medium would be suitable even for these cold-sensitive bacteria.

All the strains gave more than 300 and less than 30 colony forming units at dilution 10^{-4}, 10^{-5} and at dilution 10^{-7}, respectively, when incubated up to 4 h in ice chest.

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