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TELLURIC PSEUDOMONADS METABOLITES INVOLVED IN THE ANTAGONISM TO PHYTOPATHOGENIC FUNGI

Samia Mezaache-Aichour, Nora Haichour, Abdelhadi Guechi & Mohamed M. Zerroug
Laboratory of Applied Microbiology, Faculty of Life and Natural Sciences,
University Ferhat Abbes Sétif 1, Sétif, Algeria.

Abstract

Fourteen fluorescent *Pseudomonas* spp. potentially produce secondary metabolites involved in antagonistic activity. Among the produced secondary metabolites, some substances, as siderophores, hydrolytic enzymes and HCN with antibiotic activity provides a major advantage in the microbial antagonism to the producing strain. All strains synthesized siderophores, some strains have a single type of siderophore, while others have several siderophores. Production of proteolytic enzymes (which may be involved in parasitism) was found in only three of the strains studied. While Cyanogenesis, was found in four strains. This metabolite is a product of secondary metabolism, which may benefit the production strains in the rhizosphere microbial communities. These features, in addition to those mentioned above, could benefit the strain in its competition and its antagonistic activity in diverse ecosystems.

Keywords: *Pseudomonas*, secondary metabolites, antagonism.

1. Introduction

In soil, *Pseudomonas* represent a large fraction of the microbial community sharing environment with commensals mainly represented by the genera of *Bacillus* and *Actinomyces* . They are found everywhere, especially in the root systems of plants. Different Pseudomonas species that colonize the rhizosphere have several inherent characteristics which make them particularly attractive for use as biological control agents. Their ability to colonize the roots and maintain a high population density is remarkable (Haas and Keel, 2003). This rhizo-competence is due to their growth which is higher than that of most other rhizobacteria, and their ability to metabolize the compounds of root exudates (Chin- A- Woeng *et al*., 2003). Moreover, these bacteria are very easy to isolate and to cultivate in the laboratory, and are readily adaptable to genetic manipulations (Chin- A- Woeng *et al*, 2001). *Pseudomonas*, primarily *P. fluorescens*, has long been known for their ability to reduce the incidence of root disease in certain fields, and to inhibit the growth of many plant pathogens *in vitro.* This inhibitory capacity may be based on several mechanisms including the production of a wide range of antagonistic metabolites as siderophores. These allow it a great competition for the acquisition of iron. In an environment like the soil where this element is present in very small quantities, it can affect the saprophytic growth of several pathogens and reduce the severity of the disease; and allow some strains an ability to induce defense mechanisms in plants (Iavicoli *et al*., 2003; Kim *et al*., 2004). However, in most cases of inhibitions, the determining factor is the production of antibiotics that act directly on the pathogen. The first report on the use of antibiotic-producing Pseudomonas for the control of a root pathogen, *Rhizoctonia solani*, came from Howell and Stipatovic (1979). These authors identified pyrrolnitrin produced by a strain of *Pseudomonas fluorescens* as a factor to inhibit the pathogen. Unlike primary metabolites, secondary metabolites are not essential for growth or multiplication. But can play an important role in interactions between *Pseudomonas* species and / or with other genera, particularly during biocontrol and pathogenesis (Lau *et al*., 2004).

In this study we investigated some *Pseudomonas* isolated from potato rhizosphere activities involved in bacteria-phytopathogenic fungi interactions: (i) production of siderophores, organic ligands that complex iron, providing a competitive advantage to producing organisms in the rhizosphere, an oxygen- and iron-depleted environment, (ii) cell wall degrading enzymes involved in parasitism and / or lysis of fungi (iii) production of hydrogen cyanide (HCN), a secondary metabolite which can inhibit the growth of rhizosphere inhabiting bacteria and fungi, and sometimes interfere with root growth.

2. Material and Methods

2.1 Biological material

Strains of Pseudomonas spp. producing a fluorescent pigment on King B medium as previously described by Mezaache-Aichour *et al*. (2012) were studied for their ability to produce some secondary metabolites such as siderophores, enzymes degrading the cell walls (CWDE : enzymes with proteolytic activity and chitinases) . On the other hand, by production of antibiotics such as hydrogen cyanide (HCN). In addition, *P. protogens* CHA0 was gifted by Dr HAAS D. (Lausanne, Switzerland) was used as control, Fusarium oxysporum f. sp. albedinis (FOA, causal agent Bayoud disease of date palms) obtained from INRA Algiers, Algeria was used for the test of antagonism.

2.2 Production of secondary metabolites

2.2.1 Siderophore synthesis

Detection of siderophores was performed in the O -CAS areas (overlayed chrome azurol S) in Petri dishes .according to the modified method described by Pérez- Miranda et al. (2007). The CAS medium, containing agarose instead of agar is prepared according to Schwyn and Neilands (1987). For this, bacteria are grown on succinate agar

(Meyer and Abdallah, 1978) or on casein hydrolyzate agar (CAA, Serino et al., 1995) for 18 h at 28ºC. Detection of siderophores is accomplished by coating the bacterial culture of 10ml modified CAS medium,and based on the color changes of the medium (Schwyn and Neilands , 1987). The estimation of siderophores production was performed according to Jacques *et al*. (1995). The growth rate is estimated by spectrophotometer at 540 nm, where absorbance unit equals 5.10^8 UFC. After centrifugation at 4000 rpm (15min at 4ºC), the absorbance of the supernatant is determined at 400 nm (Spectrophotometer: JENWAY 6300), and the amount of siderophore calculated using the molar absorbance coefficient ($\varepsilon = 27000\ cm^{2.}\ mmol^{-1}$) as described by Jacques et al. (1995). Supernatants obtained to quantify siderophores were sterilized by filtration (0.22 µm). Have been subjected to chemical analysis to determine the nature of siderophores (Baakza *et al*., 2004).

2.2.2 Enzymes degrading cell walls (CWDE)

2.3.4.1 Proteolytic enzymes

The proteolytic activity was determined by the method of Smibert and Krieg (1994), by culturing isolates on agar skim milk as described by Naik and Sakthivel (2006).

2.3.4.2 Chitinases

The chitinolytic activity is estimated as described by Reniwick et al. (1991) on agar supplemented with chitin. Formation of clear zones around the colonies after 5 days of incubation at 30°C indicates a positive chitinase (Naik and Sakthivel, 2006).

2.2.3 Production of volatile antibiotic substance (HCN)

The Cyanogenesis was evaluated on liquid and solid media. The first was carried out according to the method described by Meena *et al*. (2001), where the bacterial isolates were grown for 48 at 28 ° C under continuous shaking (180rpm) in Erlenmeyer flasks containing 50 ml of TSB. Strips of 0.5cm in large and 10cm in long of Whatman N° 1 saturated with a solution of alkaline picrate , are suspended vertically in the Erlenmeyer flasks . The paper color changes according to HCN production (Verma *et al*., 2007). The second method was adapted, from Lorck (1948). On nutrient agar supplemented with 4.4g / l of glycine , bacterial isolates are inoculated by streaking, a disk of Whatman N° 1 saturated with alkaline picrate are placed in the boxes lids, they are sealed with parafilm and incubated inverted at 28 ° C for 4 days (Ahmad *et al*., 2008; Trivedi *et al*., 2008). The HCN production is proved upon the color change of the paper (Trivedi *et al*., 2008). In both cases, three repetitions were performed; the negative control is represented by a medium without inoculum, whereas the positive control is represented by the reference strain of *P. protogens* CHA0.

2.3 Antagonism

After purification of the siderophores as described by Serino *et al*. (1995), they were used for cross utilization by competitive bacteria as described by Jacques *et al*. (1995).

2.3.2 Antagonism by HCN production

Fungal antagonism by the production of volatile substances was performed as described by Trivedi et al. (2008). Observations are recorded after 24 to 120 h of incubation. Inhibition of fungal growth is calculated using the following formula, where r1 is the radial growth of the fungus control and r2 is the radial growth with bacterium (Trivedi *et al*., 2008)

$$\text{Percent inhibition} = \frac{(r_1 - r_2)}{r_1} \times 100$$

3. Results

3.1 Production of metabolites

All strains already selected on the basis of the synthesis of a diffusible fluorescent pigment on KB medium showed an orange clear halo on the - O CAS medium indicating siderophores production (Fig. 1). The studied strains produce different types of siderophores (Table I), which were determined on MS medium in the absence and in the presence of 100µM of FeCl3. While most of the strains produce hydroxamate siderophores, some isolates 5 of 6 produce catecholates, and four of six produce carboxylates ones. Regarding to the enzymes studied, there was a total lack of production of cellulases, while enzymes such as fungal cell wall proteases and chitinases (Fig. 2) are produced by strains which also produce phosphatases (Mezaache, 2012). Four of the strains tested are producing hydrocyanic acid, with variable rates. The production will be intensified after 48h, revealed by the turn of the color paper for some strains which became almost red.

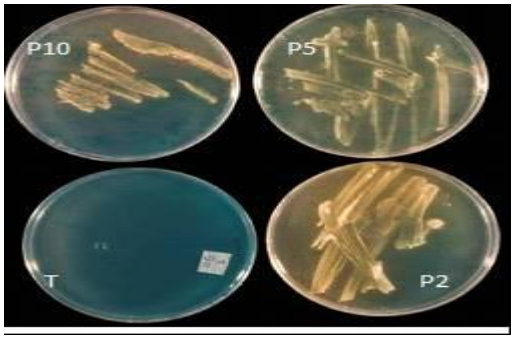

Figure 1: Siderophores on O-CAS succinate produced *by Pseudomonas* spp.
T: control; P2 P5 and P10: tested isolates

Table I: Siderophores produced by tested *Pseudomonas* spp.

Isolates	Hydroxamates	Catecholates	Carboxylates
P 1	+	-	-
P 2	+	+	+
P 5	+	+	-
P 7	+	+	+
P 8	+	+	+
P 10	+	+	+

+ and -: positive and negative production; P1, P2, P5, P7, P8 and P10: tested isolates.

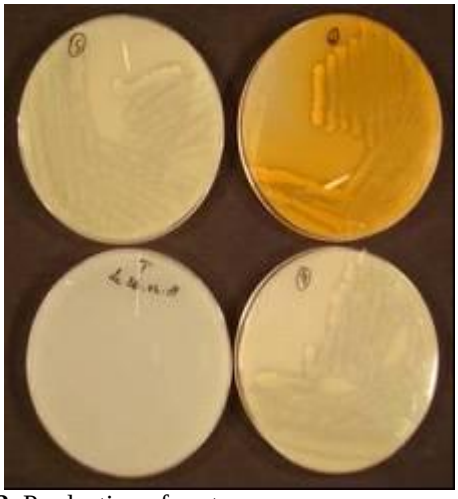

Figure 2: Production of proteases
T: control; 2, 5 and 7: tested isolates

3.2 Antagonism

Besides isolate 2 which produces PCA (Mezaache-Aichour *et al*., 2012 and 2013), the other isolates express their antagonistic activity by the production of other metabolites. Indeed, fungal antagonism observed by production of volatile substances was found in the strains producing substantial quantities of HCN. This antagonism is important in the strain CHA0 and decreasing from isolates P2, P1, P8, and finally P7 on TSA medium supplemented with glycine, and almost lower on King B medium (Fig. 3).

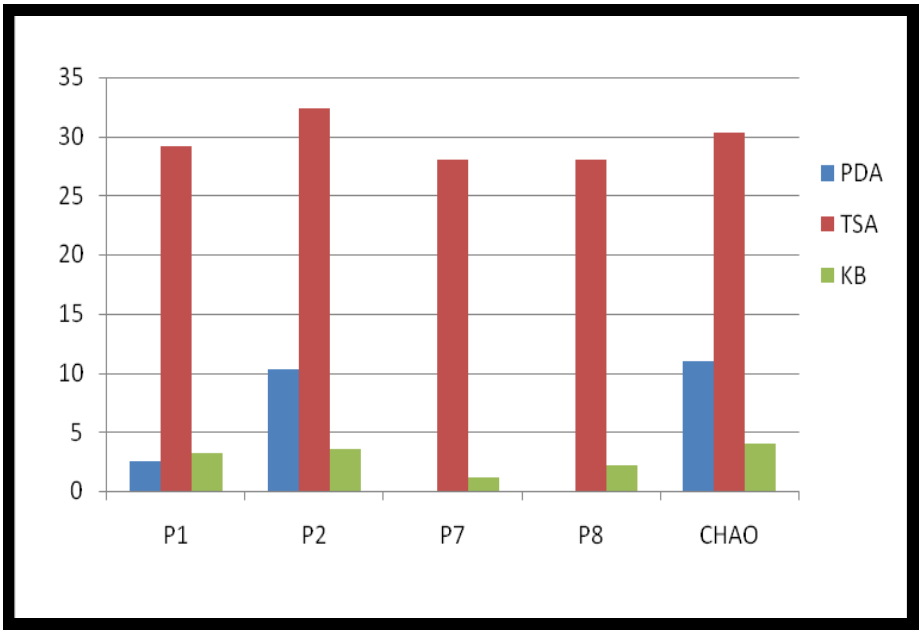

Figure 3: Antagonism by HCN production
P1, P2, P7, P8 and CHA0: Pseudomonas isolates; PDA, TSA and KB: media of tests.

The siderophore-producing isolates have also shown a significant level of antagonism on King B medium, thus isolates P2, P1, P5, P7, and P10 even have inhibited, in decreasing order fungal agents (Mezaache, 2012). Some studied bacteria have great ability of iron chelation. They can recognize and use siderophores produced by other strains, in the case of P2 (Fig. 4 a and b). While some isolates like P1 and P5 are not able to use the produced siderophores by other isolates (Fig. 4 b 'and f'). Indeed, some isolates using heterologous siderophores directly on the medium in which they are produced (Fig. 4 a, b , c, d, e and f) and semi -purified siderophores (Fig. 5, 2s, 2SU, C 7 and C 12).

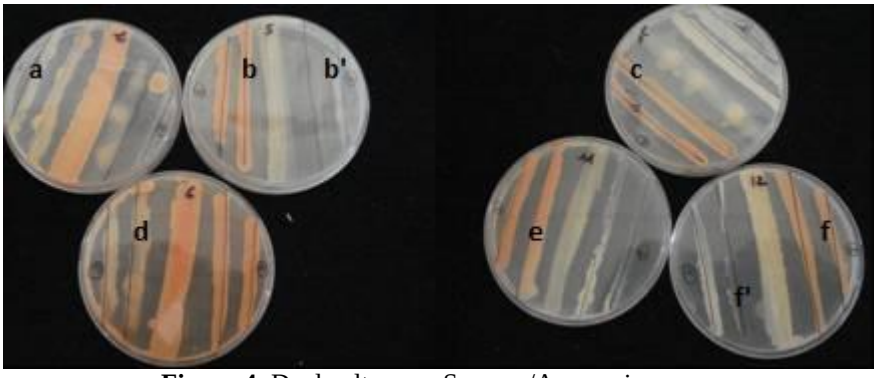

Figure 4: Dual culture on Sucrose/Asparagine agar.
a, b, c, d, e, f: isolates using heterologous siderophores; b', f': isolates can't use heterologous siderophores.

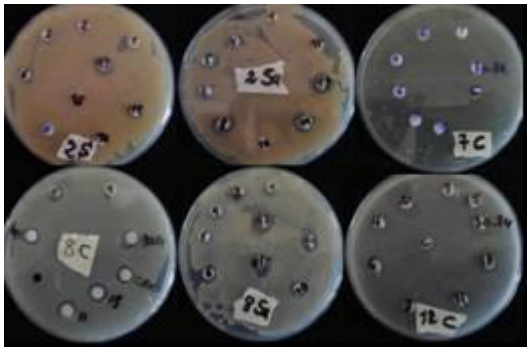

Figure 5: Cross utilization of semi purified siderophores.2S, 2Su, 8Su: isolates growing on succinate agar; 7C, 8C and 12C: isolates growing on CAA agar.

Discussion

Iron plays a crucial role in most redox enzymes involved in the intermediary metabolism electron transport chain. Despite its abundance in nature iron bioavailability is extremely low, at pH 7.0 the thresholds of ferric concentration do not exceed 10^{-18} M. To face this situation, the majority of organisms need to respond by the capture and the assimilation of iron from their environment (Dreschsel and Winkelmann, 1997). To capture and to assimilate iron, bacteria developed then complex systems capture, *via* siderophores secretion and capturing ferric siderophore complexes (Bagg and Neilands, 1987). Siderophore synthesis is only derepressed when the microbial cells are in iron deficient conditions (Meyer and Hornsperger 1978; Meyer *et al.* , 1990). Production of siderophores even occurs in fungi and monocotyledons in same conditions (Ratledge and Dover, 2000). Meyer and Abdallah (1978) have already reported that production was also observed in other strains of fluorescent *Pseudomonas* such as *P. aeruginosa* , *P. putida* , *P. chlororaphis* , *P. aureofaciens* and *P.syringae* .

For most examined strains, the pyoverdine synthesis was appreciable on MS medium, while the addition of 100µg/ml of $FeCl_3$ repressed this synthesis (Mezaache, 2012). The pyoverdins produced by *Pseudomonas* species contain two functional groups (hydroxamate and catecholate). The hydroxamates are produced by bacteria as well as fungi, while catecholates are exclusively produced by bacteria, and comprise two groups of the catechol and having other hydroxy functions as ligand. The carboxylates are produced by a fungal group (mucorales), and very few bacteria such as *Rhizobium meliloti* and *Staphylococcus hyicus*, binding of iron by two hydroxyl groups and one carboxyl (Dreschsel *et al*., 1995; Baakza *et al*., 2004). All isolates produce hydroxamates revealed on CAS medium (Schwyn and Neilands, 1987) and O -CAS (Perez -Miranda *et al*., 2007) and confirmed by paper electrophoresis (Schwyn and Neilands, 1987; Mezaache, 2012). While carboxylates and catecholates production were observed in some isolates only.

Parasitism and / or lysis of fungi by rhizospheric bacteria were facilitated by production of hydrolytic enzymes that degrade fungal cell walls (CWDE: cell wall dedrading enzymes). Chitinases are hydrolytic enzymes of major importance, since chitin (linear polymer of β - (1,4) - N- acetylglucosamine), is the major constituent of the cell walls of most phytopathogenic fungi (Kishore *et al.* , 2005). The chitinolytic activity of *Pseudomonas* spp. strain PUP6 (Naik and Sakthivel , 2006), and strains of *Pseudomonas* spp. (Verma *et al.* , 2007) was reported as antifungal activity. Purified chitinases of *Bacillus subtilis* AF1 (Manjula *et al*., 2004), *Serratia marcescens* (Kishore *et al*., 2005), *Serratia plymuthica* (Frankowski *et al*., 2001) are highly antagonistic against fungi. In addition to chitinases, *Pseudomonas* produces other types of lytic enzymes like proteases; PUP6 produces proteases sharing a CWDE activity (Naik and Sakthivel, 2006).

Some PGPR produce volatile antibiotics, the most important one is HCN which inhibits cytochrome oxidase of many organisms. Strains producing HCN have an alternative resistant cytochrome oxidase, and are relatively insensitive to HCN (Voisard *et al*., 1989). Hydrogen cyanide is a secondary metabolite produced by the $Gram^{-ve}$ as *P. fluorescens* , *P. aeruginosa* and *Chromobacterium violaceum* (Askeland and Morrison, 1983). HCN and CO_2 are formed from glycine (Castric , 1977) by the HCN synthetase (Castric , 1994) . Among *Pseudomonas* spp. this enzyme oxidizes the glycine in the presence of electron acceptors such as phenazine methosulfate (Wissing, 1974). *P. fluosrescens* CHA0 (now reclassified *P. protogens*) is an aerobic biocontrol agent, colonizing the roots of many plants and thus protecting them from fungal soil borne diseases (Voisard *et al*., 1994). HCN production by strain CHA0 is suppressive against black root rot of tobacco caused by *Thielaviopsis basicola* (Sacherer *et al*., 1994). Deffective strains of CHA0 in the production of HCN, antibiotics and exo enzymes were unable to prevent plant diseases as was for $GacA^-$ mutant strain, which losses its ability to protect tobacco from black root rot, and CHA0 defective mutants in HCN production are less effective in the

control of take-all of wheat. The complementation of this mutant by *hcn* genes cloned from the wild strain restores the ability of biocontrol (Voisard *et al*., 1989).

For a full germination and successful penetration of root ends of their hosts, special forms of pathogenic *F. oxysporum*, require iron (Baker, 1986). The introduction of *Pseudomonas* in the soil induces suppression by production of siderophores such catechol-hydroxamate group (Teintze *et al*., 1980). Fusaria also produce hydroxamate siderophore type, but these are less effective than those produced by fluorescent *Pseudomonas* spp. Therefore under iron limiting conditions, found in alkaline soils, the pathogen is deprived of assimilable forms of iron, due to the activity of rhizo-competent fluorescent *Pseudomonas* spp. (Baker, 1986).

PGPR supressiveness activity is strongly related to the absence of sufficient amount of iron, they express their great ability by depriving pathogens of iron (Kloepper *et al*., 1980 a; Schroth and Hancock, 1982). For example, *P. putida* B10, which is suppressive of Fusarium wilt and take-all, lost its supressiveness by addition of iron, this addition represses siderophore synthesis by this bacterium (Kloepper *et al*., 1980 b). Similarly, fluorescent *Pseudomonas* spp. lacking pyoverdine production, protect the plants with less efficiency than the parental strains (Keel *et al*., 1989; Loper and Buyer, 1991).

However, under certain conditions these pyoverdins can function as diffusible bacteriostatic or fungistatic antibiotic substances, while ferric pyoverdins are unable (Kloepper *et al*., 1980b; Scher and Baker, 1982). Therefore, the producers of a powerful siderophore as pyoverdine can compete efficiently with other microorganisms that have lower affinity systems for iron chelation (Haas and Defago , 2005). Some of the studied *Pseudomonas* strains are able to use heterologous siderophores in iron deficient medium (Bakker *et al*., 1988; Jurkevitch *et al*., 1992; Mirleau *et al*., 2000). The PGPR strain *P. putida* WCS358 (Geels and Schippers, 1983) is able to use its own siderophore (pseudobactin -358), *via* a highly specific receptor (Bitter *et al*., 1991), but may also use a wide range of heterologous siderophores (Koster *et al*., 1993). While pseudobactin -358 can be used only by a small number of *Pseudomonas* (Marugg *et al*., 1989; Bakker *et al*., 1990; Raaijmakers *et al*., 1994). The P10 and P12 isolates identified respectively as *P. putida* CM 5002 and *Pseudomonas* spp. (Mezaache-Aichour *et al*., 2012), are able to use xeno-siderophores. As a factor limiting growth for most organisms, iron plays a critical role in this competition (Weinberg, 2008). Bacteria and pathogenic fungi have many mechanisms for iron acquisition from host cells. Either by secretion of siderophores, which are capable of recovering iron from any organic or inorganic material , the membrane expression of reductase or specific membrane transporters (using heme) iron chelator, and the synthesis of hydrolytic enzymes or toxins (Litwin and Calderwood, 1993). Weller and Cook (1983) had previously reported that despite the importance of siderophore production in biological control, this is probably not the only mechanism involved in the antagonism.

The antagonistic activity of *Pseudomonas* is not specific to determined plant pathogens, Isolates from the rhizosphere of potato showed a diverse antagonism against fungi affecting other plants and from other habitats such as Foa. Défago and Haas (2005) reported that with the exception of pyrrolnitrin, antibiotics produced by fluorescent *Pseudomonas* spp. show reduced selectivity compared to fungi.

Conclusion

Among the secondary metabolites production of substances with antibiotic activity provides a major advantage in the microbial antagonism to the producing strain. Fluorescent *Pseudomonas* spp. isolates showed a significant *in vitro* antagonistic activity growth of special forms of *Fusarium oxysporum*. *F. oxysporum* fsp. *albedinis* is most sensitive to the actions of these *Pseudomonas*. These results show that isolates of fluorescent *Pseudomonas* spp. selected on the basis of phenotypic, metabolic and ecological properties may be an effective biological control in a natural soil, to limit the use of inputs chemical.

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