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RESEARCH ARTICLE

THE ABILITY OF LOCAL STRAINS OF *PSEUDOMONAS FLUORESCENS* ISOLATED FROM DIFFERENT AGRICULTURAL AREA TO PRODUCE FOUR SECONDARY METABOLITES

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ABSTRACT

In this study, 24 local isolates of *Pseudomonas fluorescens* have been isolatedfrom soil of different agricultural areas during the period from January to February 2011. The bacteria were identified using biochemical tests and the isolates were tested for their ability to produce secondary metabolites. The secondary metabolites have been identified using gas chromatography mass spectrophotometry (GC-MS), which revealed the four compounds as: (E) dodec-3-ene M.W. 168.32 Dalton, (R)-2 ((s)-amino-4-methylpentanamido)- 4-methypentanoic acid M.W. 24433 Dalton, (s)-hexahydropyrrolo (1,2-a)pyrazine-14-dione M.W. 154017 Dalton, and 3-isobutylhexahydropyrrolo(1,2-a)pyrazine -1,4-dione M. W. 210.27 Dalton. The first two compounds have been isolatedfor first time.

INTRODUCTION

The search for alternatives to chemical control of plant pathogens, such as biological control has gained momentum in the recent years due to the emergence of fungicide resistance in pathogens as well as increased health concerns for the producer and the consumer, (Reddy *et al*., 2007). Fluorescent pseudomonads are ubiquitous in the environment, inhabiting soil, plants, and water surfaces; mayplayan important role in promoting plant health.Several mechanisms provide the ability of the strains of fluorescent Pseudomonas for controlling plant pathogens, including competition for iron and other nutrients excluding niche, induction of systemic resistance, and the production of antimicrobial metabolites (Raaijmakers*et al*., 1999).Many strains produce secondary metabolites, such as phenazines, pyrrolnitrin, pyoleuteorin, hydrogen cyanide, pyoverdine, siderophoreand 2,4-diacetylphloroglucinol (DAPG). These secondary metabolites or antibiotics have been implicated in plant disease control enabling the producing strain to serve as a bio control agent (Corbell and Loper, 1995; Mavrodi*et al*., 1998; Maddula*et al*., 2008; Costa *et al*., 2009; Selin*et al*., 2010). DAPG which is an important compound of the natural suppressiveness of certain agricultural soils to take all disease of wheat and black root of tobacco (Gleeson *et al*., 2010), phenazines and hydrogen cyanide, thereby inhibiting soil-borne pathogens (Neidig*et al*., 2011).The aim of this study is to isolate and identify *p. fluorescens* from soil from different areas of agricultural importance; and to identify, any secondary metabolites produced by these isolates using Gas Chromatography Mass Spectroscopy (GC-MS).

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MATERIALS AND METHODS

Soil Samples

A total of 35 samples were collected during the period from January to February 2011, from 14 different agricultural areas in Basra, Missan and Thi-Qar governorates, southern of Iraq. The samples (40 g. for each) were collected in sterile nylon bags, transportation, and examination within the same day in the laboratories of marine bacteria in Marine Science Center and plant protection dept. Agriculture College- Basra University.*P. fluorescens* strain was obtained from the Ministry of Agriculture, Department of Organic Agriculture, Iraq, and uses as a reference strain throughout the following procedures.

Isolation

Soil samples were suspended in 99 ml of sterile distilled water per gram of soil and shaken vigorously 2 min (Vortex, Heidolph-Germany). The supernatant was serially diluted in sterile distilled water. Ten-fold serial dilutions from10^{-1} to $10^{-6}$was plated on pseudomonas agar base (K.B) using spread methodplates were incubated at 30 C° for 24 h.

Identification of *P. fluorescens*

P. fluorescens was identified using biochemical tests which included: the oxidase, catalase, gelatin liquefaction, arginine hydrolase, fluorescent under UV light and growth at 4 and 42 ºC (Fig 1).

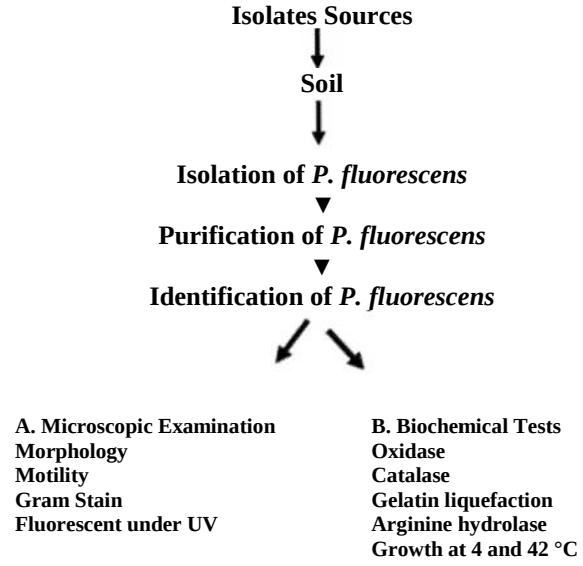


Fig. 1. Isolation and identification of *Pseudomonas fluorescens*

Production ofSecondary Metabolites

Thirty- five isolates of *P. fluorescens*were tested for their ability to produce secondary metabolites. Bacteria were cultured in KB medium at 28º C for 96h, with stirring at 120 rpm (Reddy Battu and Reddy, 2009).Secondary metabolites were purified by the ethyl acetate precipitation method (ReddyBattu and Reddy, 2009). Cells were separated from the culture broth by centrifugation at 10,000 rpm for 15 min. at 4º C. The supernatant containing secondary metabolites was left overnight with gentle stirring (40 rpm). The resulting precipitate, containing crude secondary metabolites was collected by centrifugation at 10,000 r.p.m. for 60 min at 4˚ C. Then, crude secondary metabolites were dissolved in distilled water and any insoluble contaminants were removed by centrifugation at 10,000 r.p.m for 40 min at 4 ˚C. The aqueous secondary metabolites solution was desalted by dialysis against 1Lof distilled water for 12 h with three water exchanges (Kambourova *et al*., 2001). After dialysis, the secondary metabolites solutions were lyophilized by freeze drying (Edwards, UK) to prepare pure material.

Identification of Secondary Metabolites

0.01 g of secondary metabolites was dissolved in 1 ml of methanol.200µl of each sample was diluted with 800µl methanol. 1µl was then analyzed by GC-MS (Restek-5Sil-MS, Stellenbosch University, South Africa).

Table 1. Biochemical tests of *Pseudomonas fluorescens*

District	Isolate No.	Biochemical Tests					
		Oxidase liquefaction	Gelatin hydrolase	Arginine under UV	Fluorescent at 4 ºC	Growth at 42 ºC	Growth
Basra/ Madyana	1	+	+	+	+	+	-
	2	+	+	+	+	+	-
	3	+	+	+	+	+	-
	4(M1)	+	+	+	+	+	-
	5	+	+	+	+	+	-
	6(M2)	+	+	+	+	+	-
Garmat Ali	7	+	+	+	+	+	-
	8	+	+	+	+	+	-
Al-Zubair	9	-	ND	ND	ND	ND	ND
	10	+	+	+	+	+	-
Al-Qurna	11	+	-	ND	+	ND	ND
	12	-	ND	ND	+	ND	ND
	13	+	+	+	+	+	-
	14(M3)	+	+	+	+	+	-
Al-Dear	15	+	+	+	+	+	-
	16	-	ND	ND	+	ND	ND
	17	+	+	+	+	+	-
Al-Hota	18	+	+	+	+	+	-
	19	-	ND	ND	+	ND	ND
Abu-AlKhaseeb	20	+	+	+	+	+	-
	21(M4)	+	+	+	+	+	-
Al-Hwear	22	+	+	+	+	+	-
	23	+	+	+	+	+	-
Shatt Al-Arab	24	-	ND	ND	+	ND	ND
	25	+	+	+	+	+	-
	26	+	+	+	+	+	-
Nahran Omar	27	-	ND	ND	+	ND	ND
	28	+	+	+	+	+	-
Al-Zureaji	29	-	ND	ND	+	ND	ND
	30	+	+	+	+	+	-
Al-Shafi	31	+	+	+	+	+	-
	32	-	ND	ND	+	ND	ND
Missan	33	+	+	+	+	+	-
ThiQar	34	-	ND	ND	+	ND	ND
Agriculture Ministry	35(M5)	+	+	+	+	+	-

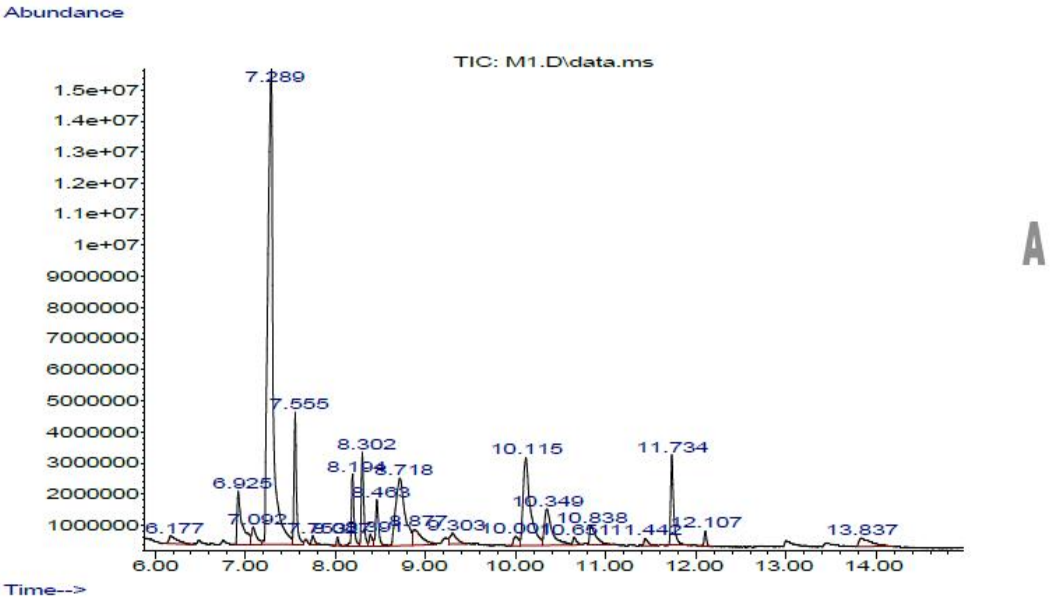
(+): Positive; (-): Negative; ND: Not Done

RESULTS AND DISCUSSION

Thirty-five soil samples were collected from 14 different agricultural areas in Basra, Missan and Thi-Qar governorates in the south of Iraq. Thirty – four isolates were isolated as presumptive *p. fluorescens*. It is noteworthy that only 24 isolates were identical in being oxidase, fluorescence under UV light (260 nm), gelatin liquefaction, arginine hydrolase, growth at 4º C positive while negative when growth at 42ºC (Table 1) (Zablotowicz *et al*., 1999; Franzettiand Scarpellini, 2007; Nathan *et al*.,2011).

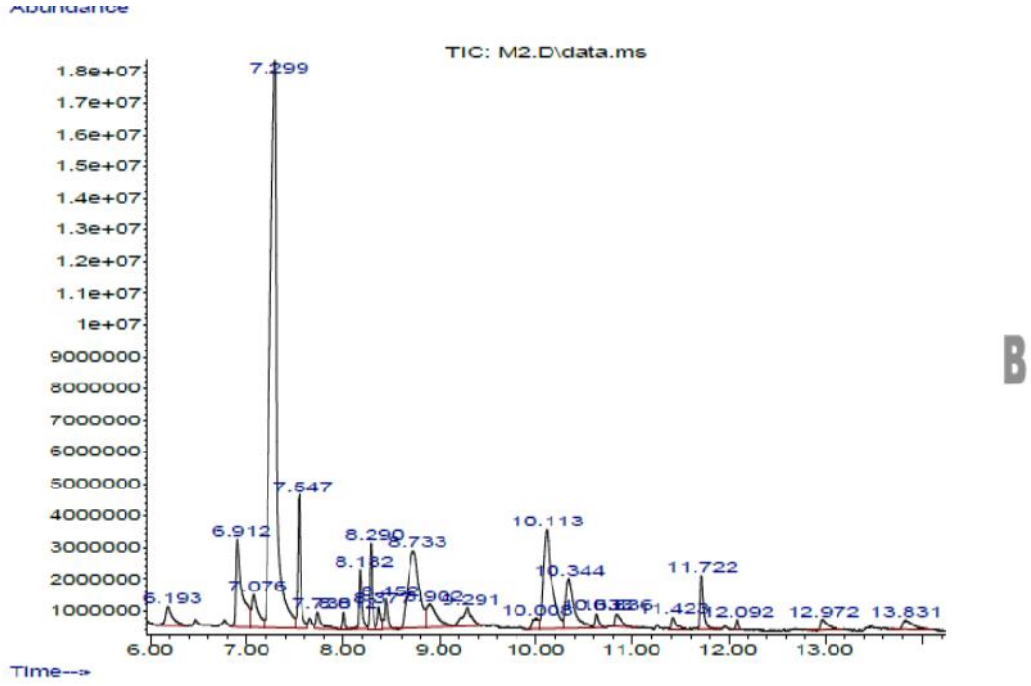
Secondary Metabolites

Four isolates of *P. fluorescens* in addition to the reference one (M1, M2, M3, M4 and M5) (Fig 3, 4, 5, 6, 7) revealed the ability to produce four secondary metabolites which including (E) dodec-3-ene M.W. 168.32 Dalton (Fig.8), (R)-2 ((s)-amino-4-methylpentanamido)- 4-methypentanoic acid M.W. 24433 Dalton (Fig. 9), (s)-hexahydropyrrolo (1,2-a)pyrazine-14-dione M.W.154017 Dalton(Fig.10), and 3-isobutylhexahydropyrrolo(1,2-a)pyrazine-1,4-dione M. W. 210.27 Dalton (Fig.11).



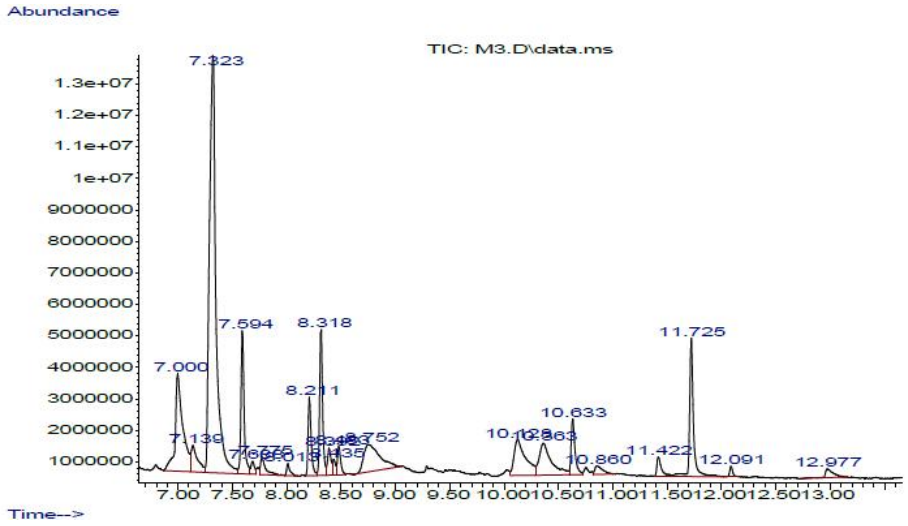
A

Fig. 3. GC- Mass analysis of secondary metabolite produce by M1



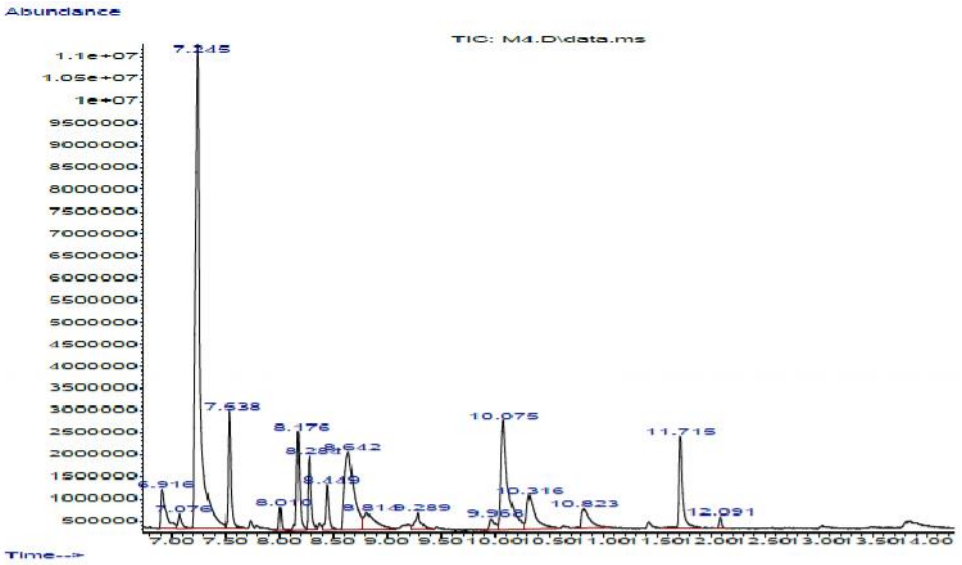
B

Fig. 4. GC- Mass analysis of secondary metabolite produce by M2



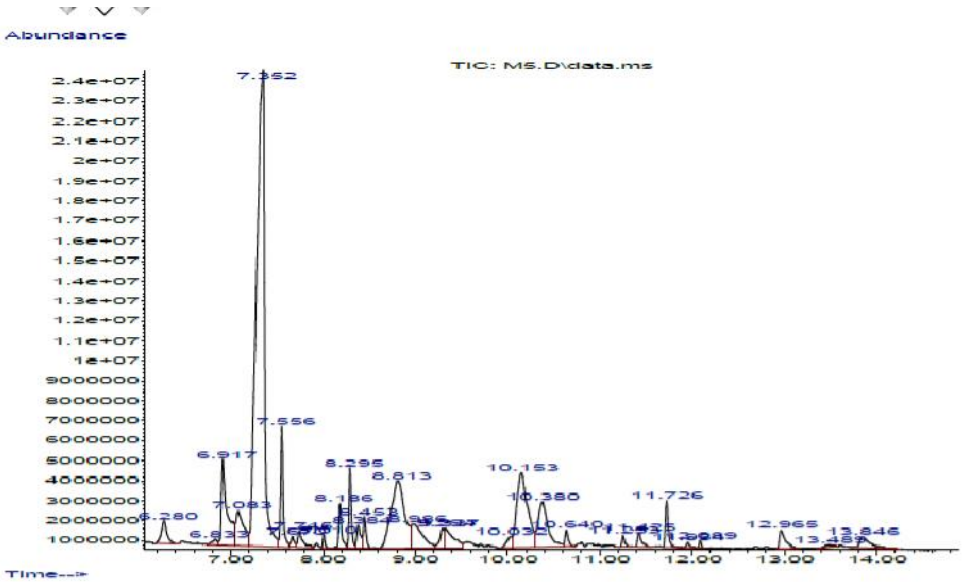
C

Fig. 5. GC- Mass analysis of secondarymetabolite produce by M3



D

Fig. 6. GC- Mass analysis of secondary metabolite produce by M4



E

Fig. 7. GC- Mass analysis of secondary metabolite produce by M5

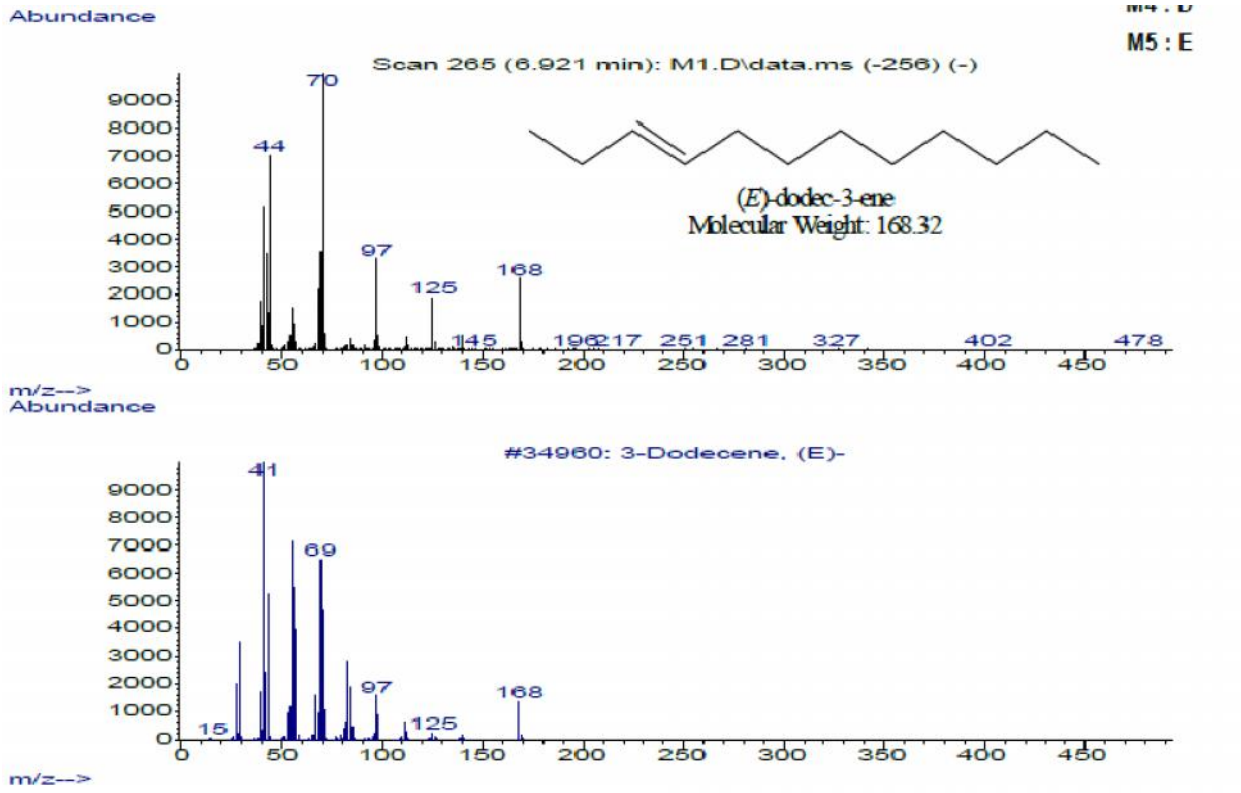


Fig. 8. GC-Mass for compound (E)- dodec-3-ene

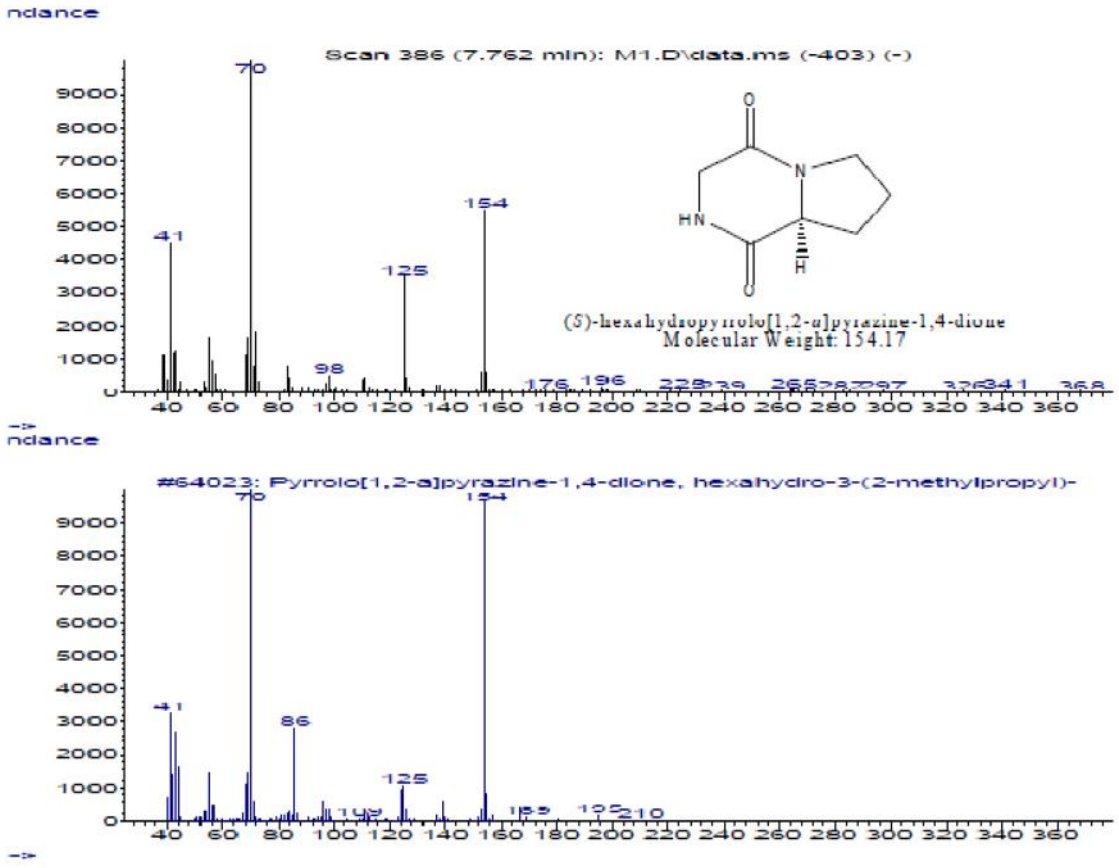


Fig. 9. GC-Mass for compound (S)-hexahydropyrrolo(1,2-a)pyrazine-1,4-dione

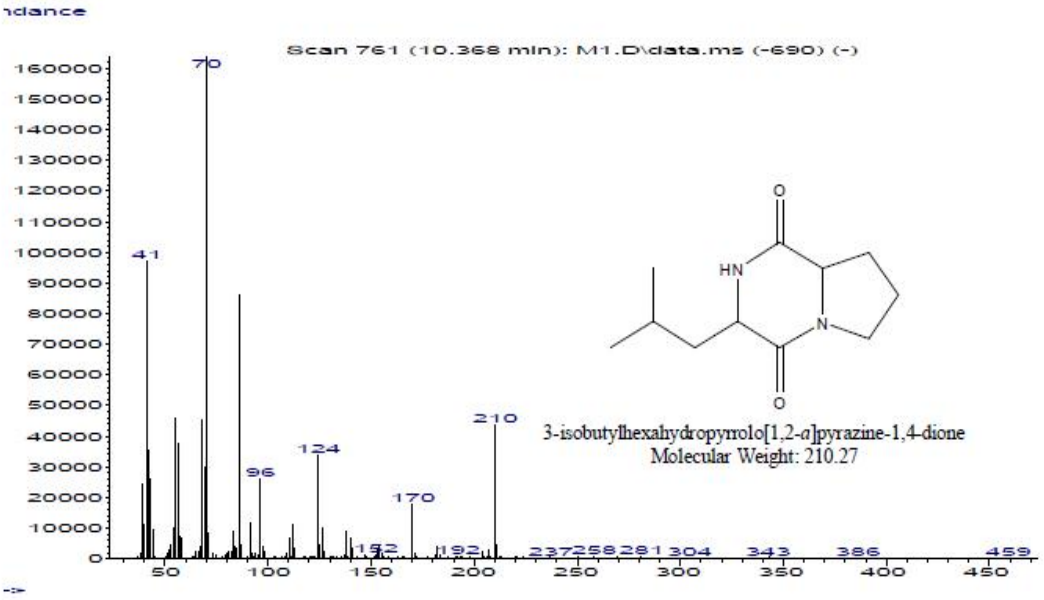


Fig. 10. GC-Mass for compound 3-isobutylhydropyrrolo(1,2-a)pyrazine-1,4-dione

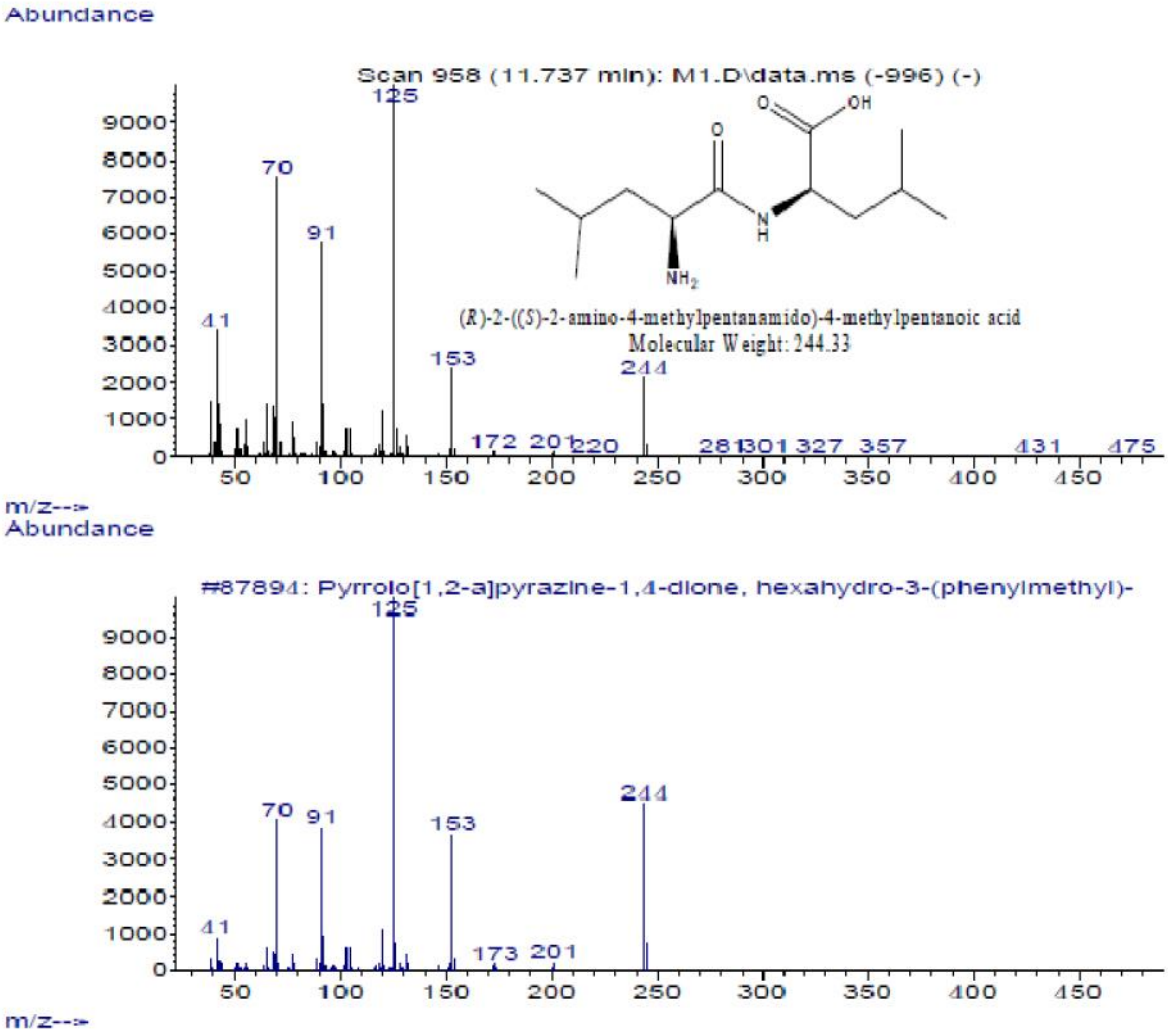


Fig. 11. GC-Mass for compound (R) -2 ((S) -2-amino-4-methylpentanamido)-4 methylpentanoic acid

From results two compounds related to pyrrol group which is an inhibitor to the growth of pathogenic fungi (Hammer *et al*, 1997; Voisard *et al*, 1989). While the other two compounds ((E) dodec-3-ene and (R)-2 ((s)-amino-4-methylpentanamido) -4-methypentanoic acid) have been isolated for the first time.

In other study (Fayyadh *et al.*, 2012) these five isolates were tested against two species of fungi *Fusarium oxysporum* and *Rhizoctonia solani*. The results of antagonism test revealed that M3 and M5 were more effective against *F. oxysporum* and *R. solani* as radial growth reach 2.7and 2.9 cm respectively comparedto 9 cm in control treatment. Additional experiment showed that bacterial isolates reduced the negative effect of the pathogenic fungi as seed germination of Okra increased from 20 and 23.3% in *F.oxysporum* and *R.solani* to 86.6% and 83.3% respectively. Results also showed that bacterial isolates were effective in reducing okra seedling damping- ofwhich reduced from 83.3 and 86.6 in F. oxysporum and *R.solani* (controltreatment) to 13.7 and 10.3 in soil treated with M3 isolate.

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