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Effect of Alkaloids Extracted from *Ruta chalepensis* L. Leaves Against Some Metabolic Activities of Sugar Beet Pathogens *In-Vitro.*

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ABSTRACT

In a previously published work, two alkaloids were isolated from *Ruta chalepensis L.* leaves firstly recorded in Egypt, and identified as furoquinoline and bis-coumarin dephnoretin alkaloids. The two alkaloids were designated throughout this work as metabolite 1 and 2, respectively. The effect of these compounds were evaluated against some metabolic activities of the sugar beet pathogens *Rhizoctonia solani*, *Sclerotium rolfsii,* and *Fusarium solani*, including cell wall-degrading enzymes, dry weight, total protein content, the free amino acid and pH values. The results showed that the rates of cell wall-degradation of cultivars *Raspoly* and *TOP* by the *in vitro* produced *R. solani* enzymes were nearly 2.5 and 5.0 times, respectively, than the other two tested pathogens enzymes produced in the absence of alkaloid stress. Under alkaloid stress, the cell wall-degrading enzymes of the pathogens supernatants were significantly decreased with increased concentration. However, the activity of *R. solani* supernatants was consistently higher than those of *S. rolfsii* and *F. solani* in all treatments. The results also revealed that the metabolite 2 was responsible for strong inhibition of the pathogens cell wall-degrading enzymes and was more effective than metabolite 1. The amounts of dry weight of the fungi studied were positively correlated with the amounts of total protein recorded in the absence of alkaloid stress. The total protein content and the amino acid pools were significantly reduced and the degree of reduction was directly proportional to the concentration of metabolite alkaloid applied. The results also, revealed considerable variations in the types and quantities of free amino acids produced by the fungi studied with referring to some of these amino acids may have a significant role in osmoregulation for the tested fungal pathogens.

Keywords: Furoquinoline alkaloid, bis-coumarin, *Ruta chalepensis,* sugar beet pathogens, cell wall-degrading enzymes, free amino acid.

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INTRODUCTION

Ruta chalepensis L.,Rutaceae (Syn. *Ruta bracteosa* D.C., *Ruta angestifolia* Pers.) is an evergreen small shrub with the common name "Egyptian rue" indigenous to the Mediterranean region, is now cultivated in many parts of the world including North America [1,2]. The leaves and roots are used in folk medicine against intestinal colic, spasmodic, amenorrhea, rheumatic diseases, headaches and wounds, skin antiseptic external treatment, insect repellent and antioxidant activity [3-6].

Rues active ingredients have antifungal and insecticidal properties that could prove beneficial to agriculture as well [7,8]. Previous phytochemical investigation of this plant resulted in the isolation of a number of alkaloids and coumarins [9-13].

Sugar beet (*Beta vulgaris* L., Chenopodiaceae) is one of the most important crops grown mainly in the areas of temperate climatic conditions for sugar production. It has great economic importance in Egypt [14]. Since, it is the second crop plant for the sugar production after sugar cane. Sugar beet is attacked by several root-rot diseases, the most serious of which are caused by *Rhizoctonia solani* Kühn (AG_{2-2}) and *Sclerotium rolfsii* Sacc., also; it is attacked by wilt diseases, the most serious of which is caused by *Fusarium solani*.

In a previous work, two alkaloid were isolated and structurally elucidated from the leaves of *R. chalepensis* and the antifungal activity was evaluated against the growth activities of the sugar beet pathogens *R. solani, S. rolfsii* and *F. solani* and was published by Emam *et al.*[13].

The aim of the present work is to find out the antifungal effect of the isolated metabolites against some metabolic activities of sugar beet pathogens are *R. solani, S. rolfsii* and *F. solani in vitro. These* activities are cell wall-degrading enzymes, total protein, dry weight and free amino acid pools.

MATERIALS AND METHODS

Host plant:

Seeds of sugar beet (*Beta vulgaris* L.) cultivars *Raspoly* and *TOP* used in the experiments were obtained from the North Delta Sugar Company, Egypt.

Culture medium:

Sugar beet pathogens: *Rhizoctonia solani* (AG_{2-2}) Kühn, *Sclerotium rolfsii* Sacc. and *Fusarium solani* (Mart.) Sacc. were isolated from diseased sugar beet roots [13-15] and maintained on the medium composed of (gL^{-1}): dextrose, 30; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; KNO_3, 2; and 1 ml of each of stock solutions ($1gL^{-1}$) of $FeSO_4.7H_2O$, $MnSO_4.7H_2O$, $ZnSO_4.7H_2O$, thiamine and agar, 20 g [16].

Plant material:

Leaves of *Ruta chalepensis* L. (Rutaceae) were collected in the flowering stage in June 2015 from plants growing on the experimental farm of the Faculty of Agriculture, Cairo University, Giza, Egypt. Plant taxonomists in the Botany Department, Faculty of Science, Cairo University confirmed the taxonomic identification of the plant species. A voucher specimen (R.C. 30) was deposited in the herbarium of Department of Biochemistry, Faculty of Agriculture, Fayoum University, Fayoum, Egypt

Alkaloids extraction from *Ruta chalepensis L*.leaves:

Furoquinoline (5-(1,1 dimethyl allyl)-8-hydroxy- furo [2-3-b] quinolone) and bis-coumarin dephnoretin alkaloids (metabolites 1 and 2, respectively) isolated from *Ruta chalepensis* L. leaves were isolated and structurally elucidated on the basis of their spectral characteristics (NMR, UV, and MS) Emam *et al.*[13].

Effect of alkaloids on cell wall-degrading enzymes of the sugar beet pathogens:

Surface-disinfested seeds of each of sugar beet cultivars *Raspoly* and *TOP* were sown in flats of autoclaved sand, placed in the greenhouse, and watered daily. Cell walls from fresh tissues of 10-day old hypocotyls were used as a carbon source for growth and enzymes induction of sugar beet pathogens *R. solani, S. rolfsii* and *F. solani* as described by Nevins *et al*. [17]. Hypocotyls were frozen at -5°C and diluted with 10 volumes of cold 0.1 M potassium phosphate buffer (pH 7) in a blender. After centrifugation at 2000 xg for 15 min. the supernatant was removed, and insoluble materials was re -suspended in fresh buffer and again centrifuged. This washing procedure was repeated three times. The residue was blended in 10 volumes of cold acetone, and cell walls were collected by filtration through Whatman No. 1 filter paper under reduced pressure. The residue was washed three times with cold acetone and dried at room temperature (ca. 27±1°C).

Erlenmeyer conical flasks (100 ml), each, containing 30 ml of the medium as described by Bateman *et al*. [18] were amended with 0.5 g of prepared cell walls as a carbon source flasks containing this media were supplemented with a l metabolites under investigation in different concentrations ranging from 10 to 40 µg ml^{-1}mixture, autoclaved for 20 min., A 6-mm diameter agar disc bearing hyphae of either sugar beet pathogens of *R. solani, S. rolfsii* or *F. solani* from 7-day old colonies were inoculated to each flask and incubated at 28±1°C for 9 days. The initial and final pH were measured .Mycelia mats and undigested cell wall materials were remove d by filtration through layers of cheesecloth at 15000 xg for 20 min at -5°C using a Beckman J-21 B centrifuge. Five flasks were prepared for each treatment. The supernatant was used immediately as the crude enzyme source in the assay.

Enzyme assay:

The activity of cell wall-degrading enzymes produced by sugar beet pathogens *R. solani, S. rolfsii* and *F. solani* were assayed by incubating 5 mg of cell walls with 1 ml of the *in vitro* crude enzyme source and 1 ml of 0.1 M potassium phosphate buffer (pH 6.5) at 30±1°C for 3 h. Reactions were stopped by boiling for 10 min, and the protein in the reaction mixtures was precipitated by mixing neutralized samples (10 ml each) with 3 ml basic lead acetate (137 g per liter), then filtering the mixture on 3 ml sodium dihydrogen phosphate (119 g per liter) to which 1 ml $H_2SO_4$was added [19]. The mixture was filtered again to remove the precipitate. Enzyme-catalyzed cleavage of glycoside bond was followed by photometric measurements of increased reducing groups by the Nelson-Somogyi reaction described by Naguib [20] at 700 nm. Results are expressed as µg reducing $sugarsml^{-1}$medium h^{-1}.

Effect of alkaloids on dry weight determination:

The experiment was conducted by mixing amounts of the metabolites with Czapek Dox's broth in 250 ml Erlenmeyer flasks to produced fungicidal stresses of 10 to 40 µg ml^{-1}mixture of a total volume of 50 ml per flask, autoclaved for 20 min., and then cooled. Control flasks without supplementation, were also prepared. A 6-mm diameter agar disc bearing hyphae of either sugar beet pathogens of *R. solani, S. rolfsii* or *F. solani* from 7-day old colonies were inoculated to each flask and incubated at 28±1°C for 9 days. pH were determined Five flasks were prepared for each treatment and filtered under suction, the mycelia mats produced was dried to constant weight at 80±1°C and the dry weight was estimated.

Effect of alkaloids on protein content in the dried mycelia mats:

Total protein (soluble + insoluble proteins) was extracted from the mycelia mats as described by Herbert *et al*.[21]. Twenty mg of ground dry mycelium were mixed with 2 ml 1 M NaOH in a test tube and the mixture was boiled in a water bath for 5 min, followed by cooling and filling up to 10 ml with NaOH. The total protein content was estimated photo metrically at 750 nm using a Perkin-Elmer spectrophotometer Model 35 (CO33-0020) as described by Lowry *et al.* [22].Protein content were decremented in the dried mycelia mat without supplementation of alkaloids

Effect of alkaloids on free amino acids in the dry mycelia mat:

Free amino acids were extracted from the dried mycelia by grinding 50 mg in a mortar with a small amount of acid-washed sand and 70% ethanol and filtering the mixture under reduced pressure. The residue

was further extracted twice with 70% ethanol. The supernatant were cooled, reduced to a known volume by heating at temperatures ranging from 40-50±1°C *in vacuo* [23]. The free amino acids were determined by paper chromatographic technique using a solvent system of n-butanol: glacial acetic acid: water (120:30:50, v/v). The spots were developed by 2% (v/v) ninhydrin in acetone and eluted for quantitative estimations with a solution of 5 mg $CuSO_4$ in 100 ml of 75% ethanol. The amounts of amino acids were estimated photo metrically at 580 nm except for proline which was measured at 430 nm [24]. free amino acids contents were decremented in the dried mycelia mat without supplementation of alkaloids

Statistics

All measurements are the means of five replicates; the results obtained were processed by analysis of variance, and the significance was determined at the least significant difference (LSD) levels of 1 and 5% [25].

RESULTS AND DISCUSSION

The rate of cell wall-degradation of cvs. *Raspoly* and *TOP* by the *in vitro R. solani* enzymes were nearly 2.5 and 5 times, respectively, faster than the two pathogens *S. rolfsii* and *F. solani* enzymes produced in the absence of the two metabolites stress (Table 1). However, there was a marked decline in cell wall-degradation of the two cultivars by the culture supernatant of sugar beet pathogens in all treatments used but the values obtained for *R. solani* were higher than those of the other two pathogens. At all the utilized concentrations of the two purified metabolites alkaloids (10-40µg ml^{-1} culture medium), the activities of wall-degrading enzymes in culture supernatants of all tested pathogens were significantly suppressed as compared with the control (Table 1). The result showed that the metabolite 2; was responsible for strong inhibition of cell wall-degrading enzymes by sugar beet pathogens and more effective than metabolite 1 alkaloid. In cv. *TOP* and in the majority of cases, inoculation with sugar beet pathogen *R. solani* produced higher enzyme activities than did inoculation with other sugar beet pathogen *S. rolfsii* and *F. solani*.

Cell wall-degrading enzymes are implicated in the action of plant pathogenic fungi on host tissues [26]. Disease development may be affected by a factor other than cell wall composition. Treatment of the host plant with metabolic inhibitors may alter cell wall-degradation and the products may not support spore germination of pathogenic fungi [27]. Furthermore, production and activity of plant pathogen enzymes on host cell walls vary among fungal species o the same genus [28], races of the same subspecies [29], under the impact of various chemical and biological stresses [30-33]. At a concentration of 40µg/ml, all cell wall-degrading enzymes by sugar beet pathogens *R. solani*, *S. rolfsii* and *F. solani* were found to be more sensitive to metabolite 2 than metabolite 1 for both tested cultivars of sugar beet *Raspoly* and *TOP* hypocotyls *in vitro*. The pH of the cell wall amended culture media of the two sugar beet cultivars shifted towards alkalinity in *R. solani* and acidity in *S. rolfsii* and *F. solani* (Table 1).

Results showed that metabolite 2 was found to be more effective than metabolite 1.pH values of the growth medium shifted towards alkalinity in *R. solani* and high acidity in *S. rolfsii* and *F. solani* (Table 2). The pH increase in the culture medium during fungal growth of *R. solani* may have been caused by differential uptake of cations and anions. Transport of anions such as phosphates may act as hydroxide exchange system with the medium becoming more basic [34]. The rapid decline in the pH of the *S. rolfsii* and *F. solani* culture were probably due to the production of organic acids (oxalic acid and fusaric acid, respectively) for *S. rolfsii* and *F. solani* through the oxidation of carbon source [35].

Effect of the alkaloids on dry mass yield and total protein content:

The dry mass yield of sugar beet pathogens *R. solani*, *S. rolfsii* and *F. solani* was decreased with increasing metabolites 1 and 2 alkaloid concentrations (Table 2). The antifungal of the two metabolites had a significant effect at all the utilized concentrations (10to 40 µg ml^{-1}) for all the tested sugar beet pathogens as compared with the control.

The dry weight yield of the fungi under investigation were positively correlated with the amounts of total proteins recorded in the absence of the two metabolites alkaloids stress (Table 2). Values of dry weight yield and total protein content of the tested sugar beet pathogens recorded under various alkaloid stresses varied significantly from the corresponding control.

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Table 1. Effect of metabolites 1 and 2 on rate of degradation of walls of two sugar beet cultivars *Raspoly* and *TOP* by the culture supernatants of sugar beet pathogens of *Rhizoctonia solani*, *Sclerotium rolfsii* and *Fusarium solani* when grown in liquid media containing sugar beet hypocotyl cell walls as carbon source

Sugar beet pathogen	Metabolite 1 (µg ml^{-1}culture medium)					LSD		Metabolite 2 (µg ml^{-1} culture medium)					LSD	
	0.0	10	20	30	40	1%	5%	0.0	10	20	30	40	1%	5%
Reducing groups (µg 100 ml^{-1} cell wall) cv. *Raspoly*														
R. solani	34.3	18.9	12.3	**5.6**	**0.4**	**2.3**	1.0	34.3	16.1	10.1	3.1	0.0	2.5	1.2
S. rolfsii	13.7	9.6	7.2	2.1	0.2	1.9	0.7	13.7	7.5	5.1	0.6	0.0	2.1	0.6
F. solani	14.0	10.9	8.5	3.4	0.5	1.8	0.9	14.0	8.6	6.2	0.9	0.0	2.2	0.8
Final pH[a]														
R. solani	6.9	7.0	7.0	7.1	7.1	-	-	6.9	7.1	7.1	7.2	7.2	-	-
S. rolfsii	5.9	4.9	4.8	4.7	4.6	-	-	5.9	4.8	4.8	4.5	4.5	-	-
F. solani	5.3	4.8	4.6	4.6	4.4	-	-	5.3	4.7	4.5	4.5	4.3	-	-
cv. *TOP*														
R. solani	59.0	37.6	24.2	13.8	2.7	3.2	1.7	59.0	33.5	18.3	9.6	0.3	2.8	1.5
S. rolfsii	11.6	8.1	6.3	3.4	0.9	2.4	1.1	11.6	5.1	3.3	0.8	0.0	2.2	0.8
F. solani	12.0	9.3	6.9	4.3	1.1	2.6	1.4	12.0	6.0	4.4	1.1	0.0	2.5	1.1
Final pH[a]														
R. solani	6.8	6.7	6.5	6.5	6.4	-	-	6.8	6.9	6.9	7.0	7.1	-	-
S. rolfsii	3.7	3.4	3.4	3.2	3.0	-	-	3.7	3.5	3.5	3.3	3.1	-	-
F. solani	4.8	3.3	3.3	3.0	2.9	-	-	4.8	3.2	3.2	3.9	2.9	-	-

Note:

[a] Final pH of the culture media after growth of fungi at 28±1°C for 9 days; initial pH of the media was 6.8 after autoclaving. [b]Metabolite 1 named; furoquinoline alkaloid., [c]Metabolite 2 named; bis-coumarin daphnoretin.

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Table 2. Effect of metabolites 1 and 2 on dry mass yield (mg) and total protein content (mg g^{-1} dry wt.) of sugar beet pathogens *R. solani, S. rolfsii* and *F. solani* after growth in broth Czapek Dox's medium for 9 d at 28±1°C

Sugar beet pathogen	Metabolite 1 (µg ml^{-1}culture medium)					LSD		Metabolite 2 (µg ml^{-1} culture medium)					LSD	
	0.0	10	20	30	40	1%	5%	0.0	10	20	30	40	1%	5%
Dry mass yield (mg)														
R. solani	1020.3	778.4	451.1	219.3	141.2	9.4	4.6	1020.3	710.2	324	182.1	71.2	10.4	4.7
S. rolfsii	612.2	341.2	172.1	130.6	89.2	10.6	5.3	612.2	290.1	161.3	87.2	45.1	11.2	5.6
F. solani	341.2	221.2	178.6	110.3	70.1	10.9	5.8	341.2	198.3	142.1	67.4	36.2	11.3	5.9
Final pH[a]														
R. solani	7.9	7.5	7.6	7.5	7.4	-	-	7.9	7.6	7.7	7.7	7.8	-	-
S. rolfsii	3.3	3.8	3.8	3.9	4.0	-	-	3.3	3.9	3.9	3.9	4.0	-	-
F. solani	3.7	4.3	4.0	3.8	3.6	-	-	3.7	4.3	4.0	3.8	3.5	-	-
Total protein content (mg g^{-1} dry wt.)														
R. solani	258.1	157.4	139.3	107.1	92.2	7.4	3.6	258.1	126.1	89.4	56.4	45.3	8.6	3.8
S. rolfsii	174.2	132.5	117.1	78.6	43.4	9.8	5.6	174.2	112.1	76.4	51.2	39.2	10.2	5.9
F. solani	148.6	121.2	106.1	64.1	33.6	10.6	5.4	148.6	100.3	71.6	45.3	31.6	11.3	5.7

Note:

[a] Final pH of the culture media after growth of fungi at 28±1°C for 9 days; initial pH of the media was 5.8 after autoclaving.

[b]Metabolite 1 named; furoquinoline alkaloid., [c]Metabolite 2 named; bis-coumarin daphnoretin.

The total protein content were significantly reduced and the degree of reduction was directly proportional to the concentration of metabolite applied, and *F. solani* and *S. rolfsii* were found to be more sensitive to the metabolite than *R. solani*. Also, the metabolite 2 was found to be more effective than metabolite 1 when used as antifungal compounds to suppress the dry mass yield and the total protein content of the tested sugar beet pathogens. Furoquinoline and cumarine are widely distributed in the plant kingdom and are present in notable amounts in the Rutaceae family. These classes have been reported to exhibit a wide array of interesting biological activities such as inhibition of various enzymes and antifungal activities (36-37).The current data suggest that alkaloids might play an important role in the rue plant's chemical defense against plant pathogens.

Amino acid pool profile of the pathogens under alkaloid stress:

Screening of the amino acid pool of the tested sugar beet pathogens *R. solani, S. rolfsii* and *F. solani* demonstrated considerable variations in the types and quantities of free amino acids produced by the fungi studied in the absence of alkaloid stress (Table 3). The free amino acids were produced in a higher quantities were tyrosine, phenylalanine, histidine, alanine, serine, threonine and proline for *R. solani;* histidine, serine, proline, methionine and cysteine for *S. rolfsii*; tyrosine, alanine, threonine, proline, tryptophan and glycine for *F. solani* in absence of alkaloid stress. All the free amino acids secreted by the tested pathogens were significantly decreased with increasing the metabolites 1 and 2 at all the utilized concentrations 10 to 40 µg ml^{-1} as compared with control. Conversely, valine was not detected in all the fungi studied. Lysine and threonine were not detected in *S. rolfsii* but were present in the remaining tested pathogens of *R. solani* and *F. solani*. The results also revealed that the metabolite 2 was found to be more effective than metabolite 1 where metabolite 2 was responsible for strong inhibition of the total free amino acids produced (32.49%, 28.99%, and 34.57%) whereas lower inhibition in the range of 35.22%, 48.33% and 36.07% was achieved with compound 1 for *R. solani, S. rolfsii,* and *F. solani*, respectively, at a concentration of 40 µg ml^{-1}. However, *S. rolfsii* was to be more sensitive to compound 2 (28.99%) than the other two tested pathogens at 40µg mL^{-1}.

The total amounts of intracellular free amino acids of the sugar beet pathogens *R. solani, S. rolfsii* and *F. solani* were strikingly variable in the absence of two metabolites alkaloid treatment (Fig. 1). *R. solani* contained the highest amount that was significantly decreased at all the utilized concentrations of alkaloid stress (10-40) µg ml^{-1}; followed by *F. solani* pathogen. *S. rolfsii* behaved similarly although it contained the lowest amount of free amino acids of the fungi studied. Potentiality of sugar beet pathogens for producing of some free amino acids under40µg mL^{-1} alkaloid stress resulted in amino acids accumulation as organic solutes, thus increasing the osmotic potential of the fungal cell. Of the amino acid assayed , tyrosine, phenyl alanine, histidine, alanine, threonine, and proline are likely to be of significance in osmoregulation by *R. solani,* whereas histidine, serine, proline, methionine, cysteine are likely to be the most effective in *S. rolfsii.* For *F. solani,* the free amino acids tyrosine, alanine, threonine, proline and glycine are likely to play a significant role in osmoregulation [38].

Over the past few years, significant efforts have been made to evaluate the effectiveness and safety of plant extracts and/or their metabolites for use in controlling plant diseases. Screening of plant extracts allows fast detection of potential sources of new bioactive molecules that can have application in medicine or the control of agricultural pests [39-44].

In conclusion, the observed tolerance of the tested sugar beet pathogens to the metabolites alkaloid stress *in vitro* may be due, in part to increased synthesis of free amino acids by these fungi and their accumulation as organic solutes, thus increasing the osmotic potential of the fungal cell. Of the amino acids assayed, tyrosine, phenylalanine, histidine, alanine and proline are likely to be of significance in osmoregulation by *R. solani* whereas proline and histidine are likely to be the most effective in *S. rolfsii*; alanine and proline are likely to the most effective in *F. solani*. Under these conditions, the remained protein detected was not retarded.

In the future, the two isolated metabolites can be used as a starting point to provide naturally based fungicides that control sugar beet pathogens instead of the harmful pesticides like Pyradur [45].

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Table3. Effect of metabolites 1 and 2 on the amounts of intracellular free amino acids (mg g^{-1} dry wt.) in the mycelium of sugar beet pathogens *R. solani, S. rolfsii* and *F. solani* after growth in broth Czapek Dox's medium for 9 days at 28±1°C

Concentration of amino acids (mg g^{-1}dry wt.)	[b]Metabolite 1 (µg ml^{-1})															LSD	
	0.0	10	20	30	40	0.0	10	20	30	40	0.0	10	20	30	40	1%	5%
Sugar beet Pathogen			*R. solani*					*S. rolfsii*					*F. solani*				
Tyrosine	25.3	7.2	5.5	4.9	3.0	0.7	0.6	0.3	0.2	0.1	4.7	4.3	4.0	3.7	3.5	0.5	0.2
Phenylalanine	17.4	10	8.6	7	5.2	0.6	0.4	0.3	0.2	0.2	0.6	0.5	0.4	0.4	0.3	1.3	0.6
Aspartic acid	1.9	1.6	1.1	0.7	0.5	1.7	1.5	1.0	0.6	0.4	2.0	1.7	1.5	1.2	0.9	0.6	0.3
Glutamic acid	2.0	1.7	0.9	0.6	0.4	2.1	1.8	1.0	0.7	0.5	2.2	2.0	1.7	1.5	1.3	0.3	0.1
Arginine	2.2	1.8	1.4	0.9	0.7	1.9	1.6	1.3	0.8	0.6	4.3	2.8	2.0	1.6	1.4	0.4	0.1
Lysine	0.8	0.6	0.4	0.3	0.1	-	-	-	-	-	0.5	0.4	0.3	0.2	0.1	0.5	0.2
Histidine	5.3	4.8	4.4	4.0	3.9	4.1	3.9	3.5	3.2	3.0	2.3	2.1	1.9	1.7	1.5	1.7	0.6
Alanine	13.0	10.2	9.1	8.2	7	1.9	1.5	0.8	0.6	0.3	16	13	10.2	7.8	6.5	2.4	0.8
Valine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Leucine	0.7	0.7	0.4	0.2	0.1	0.5	0.5	0.2	0.2	0.1	6.6	5.1	3.4	2.0	1.2	0.6	0.2
Isoleucine	0.6	0.5	0.3	0.2	0.0	0.4	0.4	0.2	0.1	0.0	6.5	5.0	3.3	2.9	2.0	0.4	0.1
Serine	2.5	2.3	2.2	2.0	2.0	2.3	2.1	1.9	1.7	1.5	1.1	1.0	0.8	0.6	0.4	0.5	0.2
Threonine	16.1	10.2	8.3	5.6	4.5	-	-	-	-	-	10.2	7.1	5.3	3.1	2.2	1.3	0.4
Proline	9.0	7.9	6.3	5.8	6.1	2.8	2.6	2.2	1.9	1.7	11.0	9.8	7.4	6.7	6.2	1.1	0.4
Methionine	1.9	1.5	1.3	0.9	0.6	1.8	1.7	1.7	1.6	1.5	1.7	1.4	1.3	1.1	0.9	0.9	0.3
Glycine	2.6	1.4	1.1	0.9	0.7	2.4	1.3	1.0	0.8	0.6	25	17	13.1	7.0	5.2	1.3	0.5
Cysteine	2.0	1.9	1.8	1.5	1.2	1.9	2.8	2.6	2.0	1.6	1.8	1.6	1.4	1.1	0.9	1.1	0.4
Tryptophan	2.9	2.5	2.0	1.9	1.4	1.8	1.9	1.6	1.3	0.9	3.3	2.9	2.3	1.9	1.5	0.9	0.3
	[c]Metabolite 2 (µg ml^{-1})															LSD	
	0.0	10	20	30	40	0.0	10	20	30	40	0.0	10	20	30	40	1%	5%
Sugar beet Pathogen			*R.solani*					*S. rolfsii*					*F. solani*				
Tyrosine	25.3	6.9	5.1	4.5	2.6	0.7	0.6	0.2	0.1	0.0	4.7	4.0	3.5	3.4	3.0	0.9	0.3
Phenylalanine	17.4	9.7	8.4	6.2	5.2	0.6	0.3	0.2	0.1	0.0	0.6	0.4	0.3	0.2	0.1	0.3	0.1
Aspartic acid	1.9	1.4	1.0	0.6	0.2	1.7	1.3	0.8	0.4	0.2	2.0	1.5	1.3	1.0	0.8	0.7	0.3
Glutamic acid	2.0	1.6	L0.8	0.5	0.2	2.1	1.6	0.8	0.5	0.3	2.2	1.4	1.2	1.0	0.6	0.7	0.3
Arginine	2.2	1.8	1.3	0.7	0.4	1.9	1.5	1.2	0.6	0.3	4.3	2.7	1.5	1.4	1.0	1.3	0.5
Lysine	0.8	0.6	0.3	0.1	0.0	-	-	-	-	-	0.5	0.3	0.1	0.0	0.0	0.3	0.1
Histidine	5.3	4.6	4.0	3.3	2.2	4.1	3.2	3.0	2.8	2.2	2.3	2.0	1.6	1.4	1.0	0.8	0.3
Alanine	13.0	9.0	8.2	7.3	6.2	1.9	1.3	0.7	0.5	0.3	16	12.1	9.8	7.3	4.8	2.4	1.0
Valine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Leucine	0.7	0.6	0.3	0.1	0.0	0.5	0.3	0.1	0.1	0.0	6.6	5.0	4.0	1.8	3.7	2.7	1.2
Isoleucine	0.6	0.4	0.2	0.1	0.0	0.4	0.3	0.1	0.0	0.0	6.5	4.8	4.7	2.5	3.8	2.8	1.3
Serine	2.5	1.7	3.2	1.5	1.1	2.3	1.4	1.2	1.1	0.9	1.1	0.9	0.6	0.4	0.1	0.8	0.3
Threonine	16.1	9.0	7.9	5.2	4.0	-	-	-	-	-	10.2	7.0	6.8	5.9	3.2	3.1	1.4
Proline	9.0	7.5	6.0	5.2	3.2	2.8	1.9	1.5	1.4	1.0	11.0	9.5	7.0	6.3	5.3	3.3	1.5
Methionine	1.9	1.3	1.2	0.8	4.0	1.8	1.3	1.1	0.8	0.6	1.7	1.2	1.0	0.8	0.5	0.6	0.2
Glycine	2.6	1.2	1.0	0.7	0.3	2.4	1.3	0.8	0.6	0.3	25	16	6.0	5.8	4.9	4.9	2.1
Cysteine	2.0	1.7	1.6	1.2	0.9	1.9	1.6	1.4	1.1	0.8	1.8	1.4	1.2	0.9	0.7	0.8	0.3
Tryptophan	2.9	2.4	1.8	1.5	4.0	1.8	1.5	1.4	1.0	0.9	3.3	2.7	2.8	1.7	1.0	1.3	0.5

Note: - = undetected, [b]Metabolite 1 named; furoquinoline alkaloid. , [c]Metabolite 2 named; bis-coumarin daphnoretin.

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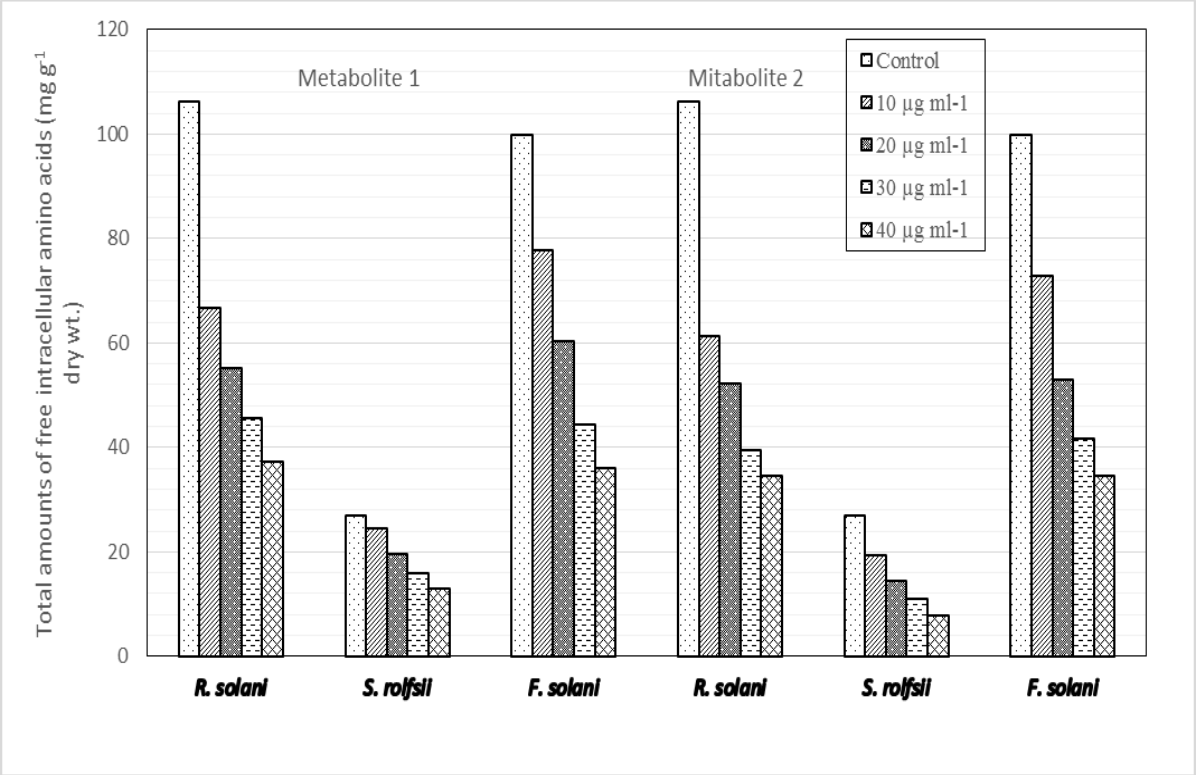

Fig.1. Total amounts of intracellular free amino acids (mg g^{-1} d wt.) produced by *R. solani, S. rolfsii* and *F. solani* after growth for 9 days at 28±1°C in Czapek Dox's medium supplemented with various concentrations (µg mL^{-1}) of metabolites 1 and 2 .

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