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Nematostatic activity of D-amino acids on the viability of root-knot nematode

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Abstract
The effect of the D-amino acids *viz.* D-Glycine, D-Methionine and D-Phenylalanine at three concentrations (100, 200 and 400 µg/mL) on the root knot nematode *Meloidogyne javanica* were estimated *in vitro* conditions. Data showed that the tested amino acids caused significantly reduction (P≤0.05) in all treatments, compared with check, where using D-Phenylalanine at 100 µg/mL conc. was recorded the best reduction in mean of second-stage juveniles hatched from egg-masses (14.3) and hatching inhibition percentage (6.24%), while treatment with D-Glycine and D-Methionine at 400 µg/mL conc. (59.33, 59.00 & 26.67) recorded the best reduction flowed by D-Phenylalanine in mean of dead second-stage juveniles and reduction mortality percentages (77.67 and 73.09%) compared with other treatments and check.

Keywords: Amino acids, root-knot nematode, *Meloidogyne javanica*, viability

Introduction
The root-knot nematodes, *Meloidogyne* spp are one of the most economically damaging genera of phytonematodes on horticulture and field crops, while phytonematodes causing global damage and loss estimated at 80 $US billion annually, root-knot nematodes alone cause 10% of loss in yield is for vegetables, to become the most common nematode species in vegetables in greenhouses, especially with the suitability of the climate and the cultivation methods in Upper Egypt, Northeast Africa ((Nicol *et al.*, 2011 [1]; Abd-Elgawad *et al.*, 2015 [2] and El-Sagheer, 2013) [3]. Recently biological methods become an attractive alternative strategy for the control of plant diseases to reduce the excessive use of agrochemicals and its health hazards. From this point of view, emphasis has been placed on amino acids as a widely present in organisms as a protein-constituting component or as substrate for methyl-transaction reactions. The use of amino acids as growth regulator provides a potential environmentally friendly and effective method of nematodes management (Zhang 2010) [4]. Also, Ton & Mauch (2004) [5] stressed that some of amino acid protects plants against a wide range of pathogens, and no harmful effects to humans or the environment have been described to date and thus it has been catalogued by the environmental protection agency of the USA (EPA 2002 [6] and Anonymous 2004 [7]), within the group of inserts that have sufficient data to substantiate that can be used safely in pesticide products. Peacock (1966) [8] and Andel (1966) [9] suggested that amino acids could be used as nematicides because of their chemotherapeutic effects on some diseased plants, also Kim and Whang (2012) [10] considered that the amino acids biochemical agent can be used as an environmentally friendly nematicidal agent of root knot nematodes. Therefore, this study aims to evaluate some D-amino acids in their pure form in effecting on root-knot nematodes as step for using in open field conditions.

Materials and methods
Amino acids preparation
The tested D-amino acids (Methionine, Glycine and Phenylalanine) was obtained from El-Gomhouria for trading chemicals and medical appliances, Egypt, and prepared the concentration, 100, 200 and 400 µg/mL of each.

Effect of amino acids on egg-masses hatching *In vitro*
The effects of tested D-amino acids on egg-masses hatching of *M. javanica* was investigated using hatching inhibition test described by Al-Sayed and Thomason (1988) [11].

Ten egg-masses in uniform in medium size, age and undifferentiated, immersed in 5 ml of three concentrations of tested amino acids at 100, 200 and 400 µg/mL prepared in sterile distilled water, on tissue paper in petri dishes (100mm x15mm), and covered to avoid evaporation and placed at room temperature (26+2°C) on laboratory bench, each treatment was replicated three replications, egg-masses in equal volumes of distilled water served as. The number of second-stage juveniles observed at 6, 24, 48, 72, 96,168 and 240 hours (Southey, 1986) [12]. Egg-masses hatching inhibition percentage and mean hatching per egg mass was calculated using the following formula:

$$\text{Mean hatch per egg mass} = \left(\frac{\text{Number of juveniles hatched in treated}}{\text{Initial no. egg} - \text{masses}}\right)$$

$$\text{Hatching inhibition \%} = \left(\frac{\text{Number of juveniles hatched in control} - \text{Number of juveniles hatched in treated}}{\text{Number of juveniles hatched in control}}\right) \text{x100}$$

Effect of amino acids on Juveniles mortality

Egg-masses were handpicked from the galled cucumber roots and incubated in sterile distilled water at room conditions at 26+2°C for 48 hours (Lee and Atkinson 1976) [13]. Hatched second stage juveniles that had passed through the tissue paper into the petri dish were counted and concentrated until reached contented 1 ml of distilled water approximately 150 second stage juveniles used each for all the treatments including the control, population density of second stage juveniles in stock suspension (150 /1ml dw) was considered mean population number from 3 times of one ml of stock suspension. 1 ml of this juveniles suspension poured in screw-capped test tubes which contained 5 ml of different concentrations of amino acids timely preparation and incubated in dark at 26+2°C for four days and the numbers of dead juveniles were counted at 12,24,48 and 96 hours (Demeure and Freckman 1981) [14], using nematode counting slide in accordance with the Baker and Hussey (1976) [15] technique modified by Boneti and Ferraz (1981) [16]. Each treatment was replicated three times, distilled water served as control the mortality percentage was calculated according to the Abbott's (1924) [17] formula.

$$\text{Mortality \%} = \left(\frac{\text{Number of survived larvae in control} - \text{Number of survived larvae in treated}}{100 - \text{Number of survived larvae in control}}\right)$$

Data analyses

All the data were subjected to Analysis of Variance (ANOVA) using Costat package version. The means were compared according to Duncan's multiple range tests at P ≤0.05.

Results

Effect of free amino acids on hatching of egg-masses of *M. javanica in vitro*

The effect of D-Glycine, D-Methionine and D-Phenylalanine at concentrations; 100, 200 and 400 µg/mL of each, on hatching of egg-masses of *M. javanica* were estimated in laboratory conditions. Data revealed that, all the tested amino acids caused significantly reduction (P≤0.05) in number of second-stage juveniles hatched from egg-masses and hatching inhibition percentage at all treatments, compared to the non-inoculated control. Data showed that, all tested martials gradual reduction in means of juveniles hatched and hatching inhibition percentages, data shown in table (1) comparing 100, 200 and 400 µg/mL concentrations of D-Glycine, D-Methionine and D-Phenylalanine, separately in each exposure time, results showed significantly differences (P≤0.05) in mean of second-stage juveniles hatched from egg-masses and hatching inhibition percentage between all treatments and control. While, D-Glycine no effect was shown after six hours of exposure in all tested concentrations, while treated with D-Methionine and D-Phenylalanine at 100 µg/mL conc. a noticeable effect appeared after 24 hours (0.7 and 2.0), with hatching inhibition percentages (11.82% and 9.1%) respectively, compared with control (3.7) to reaching after 96 hours to (3.0, 1.0 and 2.7) compared with control (29.0) to achieve hatching inhibition percentages (3.45% and 9.32%) respectively. For further confirmation due to the nature of the material the tested continue until ten days until the finishing of hatching in control to note that at 100 µg/mL conc. using D-Phenylalanine was recorded the best reduction in mean of second stage juveniles hatched from egg-masses and hatching inhibition percentage, flowed by D-Methionine then D-Glycine compared with control. And, D-Glycine, D-Methionine and D-Phenylalanine at 200 µg/mL conc. after six hours of exposure time (1.7 and 1.7), (45.95% and 45.95%) respectively, compared with control (3.7), and after 24 hours were (2.3, 0.3 and 2.7),(20.91%, 2.75% and 24.55%) respectively, compared with control (11.0), consecutively after 96 hours (4.0, 0.3 and 13.3), (13.8%, 1.04% and 45.87%) respectively, compared with control (29.0), until ten days were (139.0, 34.33 and 141.3), (60.62%, 14.97% and 61.62%) respectively, compared with control (229.3), to record that, at 200 µg/mL conc. treatment with D-Methionine was recorded the best reduction in mean of second stage juveniles hatched from egg-masses and hatching inhibition percentage, flowed by D-Glycine then D-Phenylalanine compared with control. Also, D-Glycine, D-Methionine and D-Phenylalanine at 400 µg/mL conc. after six hours of exposure time (3.3, 1.3 and 0.7), (10.19%,35.14% and 18.92%) respectively, compared with control (3.7), and after 24 hours were (2.0, 0.7 and 0.7), (18.19%, 6.37% and 6.37%) respectively, compared with control (11.0), consecutively after 96 hours (4.3, 1.3 and 5.7),(14.83%,4.49% and 19.66%) respectively, compared with control (29.0), until ten days were (42.0, 71.3 and 50.7), (18.32%, 31.11% and 22.1%) respectively, compared with control (229.3).To record that, at 400 µg/mL conc. treatment with D-Glycine was recorded the best reduction in mean of second stage juveniles hatched from egg-masses and hatching inhibition percentage, flowed by D-Phenylalanine then D-Methionine compared with control.

Evaluation of free amino acids on Juveniles development of root knot nematode, *Meloidogyne javanica in vitro*

The effect of D-Glycine, D-Methionine and D-Phenylalanine at concentrations; 100, 200 and 400 µg/mL on development as mortality of *M. javanica* were studded in

laboratory conditions. Data presented in Tables (2&3) revealed that, all the tested amino acids caused significantly reduction (P≤0.05) in the dead number of second-stage juveniles and mortality percentages at all treatments, compared to the non-inoculated control. Generally, the effect of the tested amino acids varied fluctuating between the concentrations and the period of exposure, especially during the beginning of the test, which did not provide an opportunity to draw or expect a clear line of the effect of these acids until increased period of exposure to 96 hours, so that the

Table 1: Evaluation of free amino acids on egg-masses hatching of root knot nematode, *Meloidogyne javanica in vitro*

Treatments	Mean number of juveniles hatched after intervals (Hrs.)														
	6 Hrs.			12 Hrs.			24 Hrs.			48 Hrs.			72 Hrs.		
	100µg/mL	200 µg/mL	400 µg/mL	100µg/mL	200 µg/mL	400 µg/mL	100µg/mL	200 µg/mL	400 µg/mL	100µg/mL	200 µg/mL	400 µg/mL	100µg/mL	200 µg/mL	400 µg/mL
D-Glycine	0.0 B(b)	0.0 B(b)	3.3 A(a)	0.0 B(c)	0.0 B(c)	3.7 B(b)	2.7 B(b)	2.3 B(b)	2.0 B(b)	1.0 B(b)	0.3 B(b)	3.0 B(b)	3.0 B(b)	4.7 B(b)	4.7 B(b)
D-Methionine	0.7 B(b)	1.7 A(ab)	1.3 BC(ab)	1.0 B(b)	0.7 B(b)	0.7 C(b)	1.3 B(b)	0.3 B(c)	0.7 B(c)	0.7 B(b)	0.7 B(b)	0.0 B(b)	1.0 B(b)	0.7 B(b)	0.3 B(b)
D-Phenylalanine	1.3 AB(a)	1.7 AB(a)	0.7 C(a)	0.7 B(b)	2.0 B(b)	1.3 C(b)	1.0 B(b)	2.7 B(b)	0.7 B(c)	1.0 B(b)	4.0 B(ab)	1.7 B(b)	1.3 B(b)	12.3 AB(ab)	1.3 B(b)
Control	3.7 A(a)			6.0 A(a)			11.0 A(a)			18.0 A(a)			28.0 A(a)		
LSD (0.05)	2.66	2.45	2.54	2.54	2.54	1.88	5.21	5.40	5.29	8.79	8.99	18.21	18.8	18.42	16.20

- Each figure represents the mean of three replicates.
- Capital letters represented significantly between all conc. In all treatments, and small letters represented significantly test between three conc. to alone amino acid according to Duncan's multiple-range test (P < 0.05).
- Values followed by the same letter are not statistically different according to Duncan's multiple-range test (P ≤0.05).
- Each represents mean of three replicates for three concentrations in same one period.

Continues, Table (1): Evaluation of free amino acids on egg-masses hatching of root knot nematode, *Meloidogyne javanica in vitro.*

Treatments	Mean number of juveniles hatched after intervals (Hrs.)											
	96 Hrs.			120 Hrs.			168Hrs.			240 Hrs.		
	100µg/mL	200 µg/mL	400 µg/mL	100µg/mL	200 µg/mL	400 µg/mL	100µg/mL	200 µg/mL	400 µg/mL	100µg/mL	200 µg/mL	400 µg/mL
D-Glycine	3.0 B(b)	4.0 B(b)	4.3 B(b)	3.7 B(b)	4.3 B(b)	4.7 B(b)	3.7 B(b)	7.3 C(b)	3.0 C(b)	68.3 B(b)	139.0 AB(ab)	42.0 B(b)
D-Methionine	1.0 B(b)	0.3 B(b)	1.3 B(b)	1.7 B(b)	1.0 B(b)	2.3 B(b)	3.7 B(b)	10.0 C(b)	6.0 BC(b)	25.7 B(b)	34.33 B(b)	71.3 B(b)
D-Phenylalanine	2.7 B(b)	13.3 AB(ab)	5.7 B(b)	5.3 B(b)	31.7 A(a)	3.7 B(b)	3.0 B(b)	60.0 B(b)	22.3 B(c)	14.3 B(b)	41.3 AB(a)	50.7 B(b)
Control	29.0 A(a)			47.33 A(a)			112.33 A(a)			229.3 A(a)		
LSD (0.05)	16.96	16.25	6.74	18.0	6.33	15.17	15.17	25.23	16.59	53.70	11.84	67.31

features of the effect began to appear and be stable. Data shown in tables (3&4) comparing 100, 200 and 400 µg/mL concentrations of D-Glycine, D-Methionine and D-Phenylalanine, separately in each exposure time, results showed significantly differences (P≤0.05) in mean of second-stage juveniles dead second-stage juveniles and the mortality percentages between all treatments and control. Where, after six hours of exposure time the treated with D-Glycine, D- at 100 µg/mL showed the best results (9.33 & 8.41%,), subsequently, results significantly change after 96 hours to recorded that the D-Phenylalanine (77.67&73.09%) was the best results in reduction of second-stage juveniles mortality of *M. javanica*. And, at 200 µg/mL conc. after six hours of exposure time, D-Glycine, D-Methionine and D-Phenylalanine succeeded in reducing mean of dead second stage juveniles and mortality %, With hgist result noted in treated with D-Methionine (10.33& 9.42%) respectively. With the continuation of the same order in effect after 24 and until 96 hours were D-Methionine was caused the highest results (45.33 &34.13%). At the end of the exposure period after 96 hours, the results changed significantly, where D-Glycine and D-Methionine at 400 µg/mL showing the best results flowed by D-Phenylalanine in mean of dead second stage juveniles and reduction mortality percentages.

Discussions

Data revealed that, all the tested amino acids caused significantly reduction (P≤0.05) on viability of root-knot nematode at all treatments, compared to the non-inoculated control, corroborating by Reddy *et al.* (1975) [18] were reported that, DL-methionine and DL-phenylalanine inhibited larval hatching from egg-masses in vitro to the maximum extent. Which provided by Zhang, 2010 [4] were reported the DL-amino acids had some contact toxicity *in vitro* (Amdadul Hoque *et al*., 2014 [19]).

Our data showed that, D-Glycine gradual reduction in means of second-stage juveniles hatched from egg-masses, hatching inhibition percentage, dead number of second-stage juveniles and mortality percentages, In line with Tanda *et al*. (1989) [20] were reported that inhibitory effect on egg hatch and juvenile mortality in *M. incognita* sesame root exudates in *vitro* due to their containment seven free

amino acids including glycine (Castro *et al*. 1989 [21]). Were after six hours of exposure the means of juveniles hatched and hatching inhibition percentages until 240 hours, as similar founded by Lee *et al.*(2014) [22] and Cronin *et al.*, 1997 [23]). While, in D-Methionine, data indicated that, reduction in both exposure time and concentration, and increasing in time exposure in all concentrations of D-Methionine, cased more reduction in mean number of juveniles hatched and inhibition in hatching percentages, similar results

Table 2: Evaluation of free amino acids on egg-masses hatching inhibition percentage of *Meloidogyne javanica in vitro*

Treatments	Conc. (µg/mL)	* Hatching inhibition %									*Total hatch	Mean hatch per egg mass
		6 Hrs.	1 2 Hrs.	24 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.	120 Hrs.	168 Hrs.	240** Hrs.		
D-Glycine	100	0	0	24.55	5.56	10.72	10.15	7.82	3.31	29.8	68.3	6.8
	200	0	0	20.91	1.67	16.79	13.8	9.09	6.52	60.62	139.0	13.9
	400	10.19	61.67	18.19	1.67	16.79	14.83	9.94	6.52	18.32	42.0	4.2
D-Methionine	100	18.92	16.67	11.82	3.89	3.58	3.45	3.6	3.31	11.21	25.7	2.6
	200	45.95	11.67	2.75	3.89	2.5	1.04	2.12	8.93	14.97	34.3	3.4
	400	35.14	11.67	6.37	0	1.18	4.49	4.86	5.36	31.11	71.3	7.1
D-Phenylalanine	100	35.14	11.67	9.1	35.56	4.65	9.32	11.2	53.58	6.24	14.3	1.4
	200	45.95	33.34	24.55	22.23	43.93	45.87	66.98	53.58	61.62	141.3	14.1
	400	18.92	21.67	6.37	9.45	4.65	19.66	7.82	19.92	22.1	50.7	5.1

- (*) Reduction inhibition over the control in percentage.
- (**) Total hatch represented mean number of cumulative juveniles hatched after 240 Hrs.
- Initial number of egg-masses = 10 uniform size, age and undifferentiated.

Table 3: Evaluation of amino acids on *Meloidogyne javanica* second stage juveniles mortality percentage after six exposure time *in vitro*

Treatments	The dead second-stage juveniles after six exposure time (Hours)																	
	6 H			12 H			24 H			48 H			72 H			96 H		
	100µg/mL	200 µg/mL	400 µg/mL	100µg/mL	200 µg/mL	400 µg/mL	100µg/mL	200 µg/mL	400 µg/mL	100µg/mL	200 µg/mL	400 µg/mL	100µg/mL	200 µg/mL	400 µg/mL	100µg/mL	200 µg/mL	400 µg/mL
D-Glycine	9.33 A(a)	8.00 A(a)	8.00 A(a)	41.00 A(a)	19.67 AB(ab)	10.00 BC(b)	45.00 A(a)	14.33 B(b)	15.00 A(b)	46.00 A(a)	57.33 A(a)	29.67 B(ab)	49.33 AB(a)	42.33 A(ab)	42.67 AB(ab)	52.67 B(ab)	25.67 B(a)	59.00 A(a)
D-Methionine	3.00 B(b)	10.33 A(a)	3.67 AB(b)	4.33 B(b)	50.67 A(a)	22.33AB(ab)	13.67 B(b)	64.33 A(a)	29.00 A(ab)	16.00 B(b)	21.00 B(b)	76.33 A(a)	36.00 BC(ab)	31.33 B(ab)	58.33 A(a)	25.67 C(c)	45.33 A(b)	59.33 A(a)
D-Phenylalanine	5.00 AB(a)	4.33 B(a)	4.67 AB(a)	13.33 B(ab)	29.67 AB(a)	29.67 A(a)	38.67 A(a)	13.00 B(b)	6.73 B(b)	41.67 A(a)	29.67 AB(b)	8.67 C(c)	74.33 A(a)	41.67 A(b)	43.33 AB(b)	77.67 A(a)	26.67 B(b)	19.33 B(b)
Control	1.00 B(a)			3.67 B(b)			6.33 B(b)			11.33 BC(c)			13.00 C(b)			17.33 BC (c)		
LSD (0.05)	5.75	3.60	4.48	23.21	32.19	17.76	23.15	35.89	28.06	13.50	33.56	19.76	31.16	33.72	33.15	23.40	32.85	27.82

- Each figure represents the mean of three replicates.
- Capital letters represented significantly between all conc. In all treatments, and small letters represented significantly test between three conc. to alone amino acid according to Duncan's multiple-range test ($P < 0.05$).
- Values followed by the same letter are not statistically different according to Duncan's multiple-range test ($P \leq 0.05$)

Table 4: Evaluation of amino acids on percentage mortality of the second stage juveniles of *Meloidogyne javanica*

Treatments	Conc. (µg/mL)	Juveniles mortality percentages					
		6 Hrs.	12 Hrs.	24 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.
D-Glycine	100	8.41	38.75	41.30	39.10	41.75	42.97
	200	7.07	16.60	37.38	51.87	33.71	10.44
	400	7.07	6.57	9.28	20.68	34.03	51.00
D-Methionine	100	20.2	0.68	7.83	5.26	26.43	10.44
	200	9.42	48.79	61.93	59.77	21.06	34.13
	400	2.6	19.37	24.22	19.92	72.79	51.00
D-Phenylalanine	100	4.04	10.20	34.54	34.21	70.49	73.09
	200	3.36	26.00	7.15	20.68	32.95	11.29
	400	3.70	26.00	0.42	2.99	34.86	2.41

- Initial number of juveniles = 150 second stage juveniles; fresh hatching and undifferentiated.

reported by Zhang (2010) [4] who showed that DL-methionine solutions have a direct effect on the activity of *Belonolaimus longicaudatus*. There was a concomitant increase in *B. longicaudatus* immobility with increases in the methionine concentration of solutions. It's noted by Talavera and Mizukubo, (2005) [24] were reported that after seven days in methionine solutions, the proportions of hatched eggs were reduced by 23.3% at 0.25 mg/L methionine and by 76.4% at 25 gL/1, and percentage of active *M. incognita* juveniles was reduced by 16.3 when compared with controls in water. Generally data showed that, in D-Phenylalanine as aqueous solution starting effects after six hours of exposure in all concentration for this, D-Phenylalanine conceder the fastest amino acids effecting on

hatching of the root knot nematode, compatible with Barbosa *et al.* (1999) [25]. Finally the effect of amino acid on root-knot nematode may be due to direct toxicity or the nematodes may be affected by the pH and/or osmotic pressure of the solution. (Dropkin *et al.*, 1958 [26]; Oewenberg *et al*. 1960 [27] Ahmed and Khan, 1964 [28]; Reversat, 1975 [29]; Arrigoni *et al*. 1979 [30] and Clarke *et al.* 1978 [31]). Which confirmed by Evans and Trudgill (1971) [32] were suggested that amino acids are always in the form of D-isomers sometimes serve as antimetabolites in nematodes, which could compete with these essential compounds on enzymes. While, previous research suggests that amino acid "antimetabolites" and chemical analogues of methionine, such as D-methionine or DL-methionine, "would interfere with essential intracellular metabolic pathways and enzymes in nematodes" (Crow *et al.*, 2009) [33]. While entomologists suggested the effect of amino acids is due to the ability to block ion flow through the cation-anion modulated amino acid transporter with channel properties ($CAATCH_1$) system, (Quick & Stevens, 2001 [34] and Boudko *et al.*, 2010 [35]).

Conclusion
Currently, on a global scale, it is recommended to limit the use of chemical pesticides in combating plant pathogenses, including phytonematodes and the use of alternative materials. Therefore, this study recommends using the amino acids in various forms especially that is work in dual mechanisms in terms of raising plant resistance and reducing the damage to phytonematodes and employed it within the integrated pest management and crop management programs.

Conflict of interest
The authors declare that they have no competing interests.

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Data Availability
Availability of data and materials not applicable in this study.

Ethics approval of human data or animal tissues
Not applicable in this section.

Consent for publication
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