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Tables: 3, Fig : 1

Amino acids in watermelon root exudates and their effect on growth of *Fusarium oxysporum* f.sp. *nevium*

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ABSTRACT

Watermelon cultivars resistant or susceptible to fusarium wilt were grown hydroponically as seedlings or grafted onto calabash or pumpkin rootstocks. To investigate disease resistance, the composition and content of amino acids in root exudates were analysed using an automatic amino acid analyser. Fifteen amino acids were detected in the exudates. Amino acid content was lowest in resistant cultivars and highest in susceptible cultivars, but there were no significant differences between the two rootstocks. The amino acid content was significantly higher in root exudates from resistant cultivars grafted on calabash rootstock than those from watermelon seedlings, whereas, susceptible cultivars grafted on pumpkin rootstock had lower amino acid content than watermelon seedlings. The effects of different amino acids concentrations (0, 0.6, 1.2, and 1.8 $g \cdot L^{-1}$) on growth and development of *F. oxysporum* f.sp. *nevium* were measured. Asparagine, methionine, leucine, glutamic acid and phenylalanine promoted the growth and development of *F. oxysporum* f.sp. *nevium* at concentrations of 1.2 $g \cdot L^{-1}$ or more; but methionine and phenylalanine inhibited the growth and development of *F. oxysporum* f.sp. *nevium* at conc of 0.6 $g \cdot L^{-1}$ or less.

Key words: Amino acids, *Fusarium oxysporum* f.sp. *nevium,* root exudates, watermelon.

INTRODUCTION

Watermelon (*Citrullus lanatus* (Thunb.) Matsumara & Nakai) is major crop. Its fusarium wilt (*Fusarium oxysporum* f.sp. *nevium*) has become a worldwide soil-borne disease, however it was first found in North Carolina, USA. It severely reduces the yield of watermelon and even leads to no yields in soil continuously planted with watermelon. It has been suggested that grafting of watermelon enhances the resistance to fusarium wilt and increase the yields (6,8,10,11). Cucurbitaceous crops, for example calabash (*Lagenaria siceraria* (Molina) Standl.) or pumpkin (*Cucurbita moschata* (Duch.) Poir.), which is highly resistant to Fusarium wilt is used as grafting rootstock in watermelon cultivation.

Buxton (2) showed that differences in biochemical characteristics between resistant and susceptible cultivars influenced the crop's disease resistance, which may be reflected in root exudates and the composition of root exudates differed between the crop cultivars. The root exudates of different crops contain different types and quantities of

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amino acids and organic acids (1,7,19,21). The amino acid content in root exudates has been determined in cotton (12,13,20) and eggplant (14). In this study, we selected one resistant cultivar and one susceptible cultivar from 12 watermelon cultivars. The chosen watermelon cultivars were used as scions and were grafted onto calabash and pumpkin rootstocks. We investigated the amino acid composition of root exudates and the effects of amino acids on growth and development of *Fusarium oxysporum* f.sp. *nevium*. The objective was to clarify the relationship between the watermelon root exudates and fusarium wilt. It would be beneficial to do further researches about the relationship between autointoxication and continuous cropping problem.

MATERIALS AND METHODS

We obtained *Fusarium oxysporum* f.sp *niveum* cultures from Pathology Laboratory of our University. The watermelon cultivars used and the seed sources were: ‘Zaokangjiali’, ‘Tianniu’, ‘Tianshi’, ‘Xiaowangzi’, ‘Zaokangjingxin’, ‘Jingxinyihao’ (Hefei Fengle Seed Company Limited); ‘Nongxuehuangrou’, ‘Heituleiwang’ (Harbin Xingnong Seed Company Limited); ‘Zaochunhongyu’, ‘Heimeiren’ (Harbin Kenfeng Seed Company Limited); ‘Beileerhao’ (Beijing Jinzhong Huinong Agricultural Technology Development Company Limited); and ‘Lipinyihao’ (Sichuang Zhongdu Seed Company Limited). Calabash ‘Tiezhenyihao’ (Longda Seed Company Limited, Changyi, Shandong) and pumpkin ‘Derite’ (Tianjing Deruite Seed Company Limited) were used as rootstocks. Experimental treatments were: I. Watermelon ‘Tianniu’ ungrafted seedling, II. Watermelon ‘Tianshi’ ungrafted seedling, III. Calabash ungrafted seedling, IV. Pumpkin ungrafted seedling, V. Calabash (rootstock) + ‘Tianniu’ (scion) grafted seedling, VI. Calabash (rootstock) + ‘Tianshi’ (scion) grafted seedling, VII. Pumpkin (rootstock) + ‘Tianniu’ (scion) grafted seedling, VIII. Pumpkin (rootstock) + ‘Tianniu’ (scion) grafted seedling.

Resistance of the watermelon cultivars to fusarium wilts

To evaluate the resistance of different watermelon cultivars to fusarium wilt, the dip-root subculturing method was used. Seeds of watermelon cultivars were rinsed in sterilised water, then soaked in water at 55 °C for 15 min and then at 30 °C for 12 h. After rinsing several times with sterilised water, the seeds were germinated in the dark at 30 °C. The seedlings were transplanted to a sandbox when the radicles were 0.5 cm long. Prior to use, the sand was sterilized at 100 °C and rinsed in sterilised water. Subculturing was performed during expansion of the first euphylla. The seedlings were rinsed in sterilised water and soaked in a *Fusarium oxysporum* f.sp *niveum* spore suspension (1×10^6 spores mL^{-1}) for 30 min. Subsequently, the subcultured seedlings were planted in sterilised soil and cultivated in a greenhouse at 25~28 °C. The disease incidence rate was assessed 15 days after cultivation and the disease index (DI) were calculated. The DI indicates the severity of disease and was calculated by following formula:

$$DI = (A\times0 + B\times1 + C\times2 + D\times3 + E\times4) \times 100/4 \times (A + B + C + D + E)$$

Where, A: Number of diseased plants of grade 0; B : Number of diseased plants of grade 1; C: Number of diseased plants of grade 2; D: Number of diseased plants of grade 3; and E : Number of diseased plants of grade 4.

The grades of disease severity were Grade 0 = Seedlings grew well and showed no disease symptoms; Grade 1 = Slight disease symptoms appeared in the seminal leaves but the whole plant grew well; Grade 2 = The plant exhibited slight wilting or one seminal leaf was etiolated; Grade 3 = The plant suffered moderate wilting and the seminal leaves showed drooping or rigidity and were strongly recurved and Grade 4 = The plant exhibited serious wilting, lodging and withering.

The criteria for classification of resistance were as per standards (3,4) of National Watermelon Anti-disease Seed Breeding Cooperation. Thus, based on their DI values, cultivars were classified as (i). Highly resistant (DI 0–20), (ii). Moderately resistant (DI: 21–50), (iii). Slightly resistant (DI: 51–80) (iv). Susceptible (DI: 81–100).

Collection and treatment of root exudates

The collection and treatment of root exudates were done as per method of Han *et al*. (7). The seeds of each watermelon cultivar and rootstock were rinsed three times in sterilised water, then stirred in water at 55 °C for 15 min. When the temperature was constant, the watermelon, calabash and pumpkin seeds were soaked for 10 h, 12 h, and 6 h, respectively. After rinsing, the seeds were germinated at constant 30 °C in a growth cabinet. When the radicles were 0.5 cm high, the seedlings were planted in sterilised vermiculite in 50-well agricultural seedling trays. For each cultivar 500 seeds were planted.

In addition, we adopted the top-insert grafting method. One week after the calabash and pumpkin seedlings were planted, the seeds of ‘Tianshi’ and ‘Tianniu’ were soaked for germination. When the rootstock seedlings began to form euphylla and the cotyledons of watermelon seedlings were expanded, the watermelon scions were grafted onto the calabash and pumpkin rootstocks. Each treatment included an extra 300 plants to ensure an adequate survival rate of grafted plants. The surviving grafted seedlings, watermelon seedlings and rootstock seedlings were used in the experiments to evaluate the root exudate composition and resistance to fusarium wilt. When the second euphylla of all seedlings developed, the seedling trays were placed into a cultivation channel (150 cm × 100 cm × 12 cm) and added 100 L sterilized water for hydroponic culture. After 3 days the culture solutions were collected. The culture solutions were roughly filtered three times in a Buchner's filter and then filtered through membrane of 0.45 μm pore size. Finally, 200 ml samples of each culture solution were condensed by vacuum rotary evaporation to 10 ml, which were termed the concentrated root exudate solutions and were stored at -20 °C until use.

Deleted: (see Tables 1 and 2 for sample sizes)

Analysis of amino acid composition in root exudates

The concentrated root exudate solutions for each treatment were analysed with a Japan L-8800 automatic amino acid analyzer to determine the composition of amino acids. Because the amounts of five amino acids (asparagine, glumatic acid, methionine, leucine and phenylalanine) were high in the root exudates and showed some relationship between the eight treatments after analysis, solutions of each of these amino acids were prepared at concentrations of 0, 0.6, 1.2 and 1.8 $g\cdot L^{-1}$ to determine the effects of amino acids on spore germination, mycelial growth and biomass of *F. oxysporum* f.sp. ***nevium.***

Effects on spore germination, mycelial growth and biomass

Preparation of 0.6, 1.2 and 1.8 $g\cdot L^{-1}$ amino acids (asparagine, glumatic acid, methionine, leucine and phenylalanine) solutions was based on the method of Wu *et al*. (15). Sterilised water was used as control. A *F. oxysporum* f.sp. *nevium* spore suspension was prepared for each amino acid solution and two drops of the suspension were placed on each concave slide. Each treatment was replicated six times in a complete randomised experimental design. The concave slides were placed in porcelain plates with lids and incubated at 25 ºC. After 24 h, the slides were examined microscopically to determine the spore germination rate. The mean spore germination rate was used for statistical analysis.

Following the methods of Han (16) and Yang (18), 200 ml of either 0.6, 1.2 or 1.8 $g{\cdot}L^{-1}$ amino acid solutions were added to an agar culture medium (2 g KNO_3, 1 g $MgSO_4{\cdot}7H_2O$, 1 g KH_2PO_4, 30 g fructose, 17 g agar, 1 L distilled water). Sterilised water was used as the control. *Fusarium oxysporum* f.sp. *nevium* cultures of the same age were inoculated onto each amino acid culture medium. The culture plates were placed upside down and incubated at 25 ± 1 °C. The plates were observed after 72 h and 120 h culture. The length of mycelia was measured with a vernier caliper. Each treatment comprised three replicates and the mean of four measurements per replicate was calculated. Using the same culture methodology, the microbial biomass dry weight was determined after 72 and 120 h culture. There were three replicates per treatment in this experiment.

Statistical Analysis: Data were analysed using the Microsoft Excel 2003 and SAS 6.12 programs. Means and least significant differences at the 1% and 5% level were calculated using SAS 6.12 software.

RESULTS

Resistance to *Fusarium* wilt of the watermelon cultivars

'Zaochunhongyu' and 'Tianniu' were highly resistant to *Fusarium* wilt (Table 1). 'Tianshi' was a susceptible cultivar, 'Heimeiren' was slightly resistant, and the other eight cultivars studied were moderately resistant. Based on these results, 'Tianniu' and 'Tianshi' were chosen as test cultivars for further investigation.

Composition of amino acid in root exudates

Of the 17 amino acids analysed in the root exudates, all but tyrosine and proline were detected (Table 2). Valine was detected only in exudates of resistant cultivar 'Tianniu' grafted onto calabash rootstock and lysine was not found in exudates from the resistant cultivar 'Tianniu' and the susceptible cultivar 'Tianshi'. The total amino acid content was lowest in ungrafted seedlings of 'Tianniu' and highest in those of 'Tianshi'. There were no significant differences in the amino acid content of exudates from ungrafted calabash and pumpkin seedlings. In grafted seedling treatments, the amino acid content of 'Tianniu' was significantly lower than 'Tianshi'. The amino acid content in grafted seedlings of 'Tianniu' was higher than that in ungrafted seedlings of 'Tianniu', whereas, the amino acid content of grafted seedlings of 'Tianshi' was lower than ungrafted seedlings of 'Tianshi'.

Spore germination

After 24 h cultivation, the germination rate of *F. oxysporum* f.sp. *nevium* spores was significantly higher at asparagine, glumatic acid, leucine and phenylalanine

Table 1. Resistance of 12 watermelon cultivars to *Fusarium* wilt

Cultivar	Disease incidence rate	Disease index	Resistance
'Tianshi'	93.33	81.25	S
'Heimeiren'	88.33	53.75	SR
'Beile'	73.33	35.83	MR
'Nongxuehuangrou'	73.33	35.42	MR
'Xiaowangzi'	71.67	34.17	MR
'Kangbingjingxin'	68.33	40.42	MR
'Heidilei'	66.67	38.75	MR
'Zaokangjiali'	63.33	40.42	MR
'Lipinsanhao'	61.67	37.92	MR
'Jingxinyihao'	46.67	32.08	MR
'Zaochunhongyu'	38.33	17.92	HR
'Tianniu'	31.67	14.17	HR

HR = high resistance, MR = moderate resistance, SR = slight resistance, S = susceptible. Data is mean of 60 plants.

Table 2. The composition and content of amino acid in root exudates from different treatments (unit: μg /stub)

Amino acid	Treatment							
	I	II	III	IV	V	VI	VII	VIII
Asparagine	2.73	6.31	4.81	5.33	5.71	5.63	4.02	3.52
Threonine	0.38	0.24	0.20	0.22	0.23	0.33	0.41	0.42
Serine	0.31	0.20	1.50	0.20	0.61	0.23	0.18	0.24
Glutamic acid	3.14	7.63	5.91	5.04	3.34	4.52	3.62	7.71
Glycine	0.73	0.14	0.42	0.11	0.34	0.48	0.21	0.17
Alanine	0.13	0.23	0.51	0.12	0.42	0.42	0.23	0.22
Cysteine	1.12	1.80	2.60	2.93	2.54	2.16	2.42	2.43
Valine	0.94	0	1.82	0	0	0	0	0
Methionine	1.80	5.12	3.34	4.34	3.08	5.42	3.81	6.92
Isoleucine	0.21	0.24	0.20	0.29	0.25	0.13	0.31	0.11
Leucine	2.32	4.90	5.01	6.81	6.44	6.65	3.18	3.42
Tyrosine	0	0	0	0	0	0	0	0
Phenylalanine	5.61	8.21	4.89	4.92	4.39	6.33	4.19	7.37
Lysine	0	0	0.32	0.12	0.23	0.13	0.19	0.28
Histidine	2.41	2.63	3.65	3.73	4.02	4.35	3.22	2.26
Arginine	1.81	1.96	3.06	2.74	2.62	2.73	1.12	1.48
Proline	0	0	0	0	0	0	0	0
Total	23.64	39.61	38.24	36.90	34.22	39.51	27.11	36.55

I. Watermelon 'Tianniu' ungrafted seedling, II. Watermelon 'Tianshi' ungrafted seedling, III. Calabash ungrafted seedling, IV. Pumpkin ungrafted seedling, V. Calabash (rootstock) + 'Tianniu' (scion) grafted seedling, VI. Calabash (rootstock) + 'Tianshi' (scion) grafted seedling, VII. Pumpkin (rootstock) + 'Tianniu' (scion) grafted seedling, VIII. Pumpkin (rootstock) + 'Tianniu' (scion) grafted seedling.

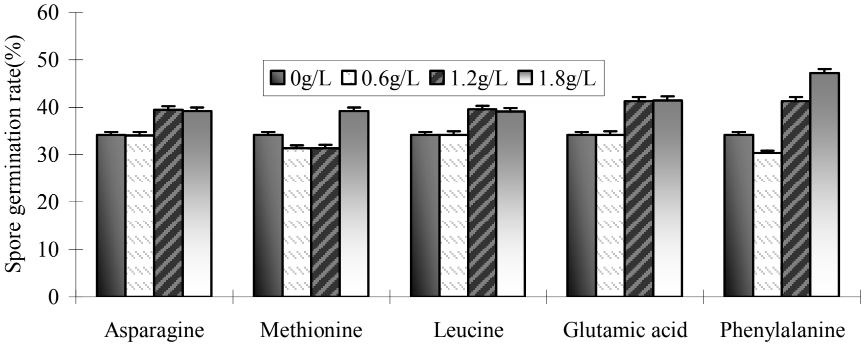

Figure 1. Effects of concentration of five amino acids on spore germination rate of *Fusarium oxysporum* f.sp. *nevium*

Table 3. Effects of concentration of five amino acids on mycelial length and biomass of *Fusarium oxysporum* f.sp. *nevium* after 72 h or 120 h culture.

Amino acid	Mycelium length (cm)		Mycelial biomass (mg)	
	72 h	120 h	72 h	120 h
Control	3.34 ± 0.06 cC	6.12 ± 0.07 cC	41.31 ± 0.27 cC	55.29 ± 0.22 dD
Asparagine $_{0.6}$	3.53 ± 0.04 bB	6.14 ± 0.06 cC	41.23 ± 0.26 cC	63.47 ± 0.35 cC
Asparagine $_{1.2}$	3.88 ± 0.05 aA	6.54 ± 0.08 bB	57.62 ± 0.41 bB	77.52 ± 0.23 bB
Asparagine $_{1.8}$	3.92 ± 0.06 aA	7.28 ± 0.06 aA	63.45 ± 0.45 aA	88.41 ± 0.31 aA
Control	3.34 ± 0.06 bB	6.12 ± 0.07 bB	41.31 ± 0.27 cC	55.29 ± 0.22 dD
Methionine $_{0.6}$	3.14 ± 0.08 cC	5.49 ± 0.07 cC	41.01 ± 0.08 cC	63.47 ± 0.35 bB
Methionine $_{1.2}$	3.34 ± 0.06 bB	5.40 ± 0.07 cC	43.38 ± 0.19 aB	59.72 ± 0.16 cC
Methionine $_{1.8}$	3.94 ± 0.07 aA	7.28 ± 0.06 aA	63.45 ± 0.45 aA	89.65 ± 0.14 aA
Control	3.34 ± 0.06 cC	6.12 ± 0.07 dD	41.31 ± 0.27 cC	55.29 ± 0.22 dD
Leucine $_{0.6}$	3.48 ± 0.06 cC	6.35 ± 0.07 cC	41.10 ± 0.02 cC	71.39 ± 0.35 cC
Leucine $_{1.2}$	3.91 ± 0.09 aA	6.61 ± 0.09 aB	57.84 ± 0.21 aB	77.72 ± 0.33 aB
Leucine $_{1.8}$	3.88 ± 0.08 aA	7.35 ± 0.08 aA	63.59 ± 0.28 aA	88.53 ± 0.32 aA
Control	3.34 ± 0.06 bB	6.12 ± 0.07 cC	41.31 ± 0.27 cC	55.29 ± 0.22 dD
Glutamic acid $_{0.6}$	3.38 ± 0.11 bB	6.18 ± 0.06 cC	41.45 ± 0.22 cC	63.76 ± 0.27 cC
Glutamic acid $_{1.2}$	4.17 ± 0.05 aA	6.71 ± 0.10 bB	57.93 ± 0.28 bB	82.32 ± 0.19 bB
Glutamic acid $_{1.8}$	3.98 ± 0.13 aA	7.28 ± 0.06 aA	63.73 ± 0.28 aA	88.41 ± 0.31 aA
Control	3.34 ± 0.06 cC	6.12 ± 0.07 cC	41.31 ± 0.27 cC	55.29 ± 0.22 cC
Phenylalanine $_{0.6}$	3.58 ± 0.04 cC	5.43 ± 0.11 dD	39.78 ± 0.10 dD	51.12 ± 0.12 dD
Phenylalanine $_{1.2}$	4.27 ± 0.13 bB	6.59 ± 0.06 bB	58.04 ± 0.10 bB	77.41 ± 0.18 bB
Phenylalanine $_{1.8}$	4.66 ± 0.11 aA	7.34 ± 0.06 aA	63.78 ± 0.20 aA	84.30 ± 0.07 aA

Figures given are the mean ± standard deviation. Capital letter indicates $P < 0.01$; small letter indicates $P < 0.05$. Means with different letters are significantly different.

concentrations of 1.2 $g\cdot L^{-1}$ and 1.8 $g\cdot L^{-1}$, than in control (Fig 1). The spore germination rate did not differ significantly from the control at asparagine, glumatic acid and leucine concentrations of 0.6 $g\cdot L^{-1}$, but was significantly lower than control and methionine and phenylalanine concentrations of 0.6 $g\cdot L^{-1}$.

Mycelial growth

After 72 h and 120 h culture, mycelial length was significantly higher than control at asparagine, methionine, glumatic acid, leucine and phenylalanine concentrations of 1.2 g·L^{-1} and 1.8 g·L^{-1}, and was significantly lower than control at methionine and phenylalanine concentrations of 0.6 g·L^{-1} (Table 3). After 72 h, there was no significant difference in mycelial length between the control and leucine and glumatic acid concentrations of 0.6 g·L^{-1}, but mycelial length was significantly higher than control at 0.6 g·L^{-1} asparagine. After 120 h, there was no significant difference in mycelial length between asparagine and glumatic acid concentrations of 0.6 g·L^{-1} and control, but mycelial length was significantly higher than control at 0.6 g·L^{-1} leucine.

Mycelial biomass

After 72 h and 120 h culture, mycelial biomass was significantly higher than control at asparagine, methionine, glumatic acid, leucine and phenylalanine concentrations of 1.2 g·L^{-1} and 1.8 g·L^{-1}, and was significantly lower than control at 0.6 g·L^{-1} phenylalanine (Table 3). After 72 h, there was no significant difference in biomass between asparagine, methionine, leucine, glumatic acid concentrations of 0.6 g·L^{-1} and the control. After 120 h, biomass was significantly higher than control at asparagine, methionine, glumatic acid and leucine concentrations of 0.6 g·L^{-1}.

DISCUSSION

Amino acids are the basic structural component of proteins (5), determine the active site of enzyme, maintain an enzyme's native conformation, and are components of coenzymes. In addition, amino acids are essential growth factor for microorganisms (9,22).

In this study, the composition of amino acids in root exudates of resistant and susceptible watermelon cultivars did not differ significantly, with 13 and 14 different amino acids detected, respectively. Yuan *et al*. (20) studied the composition and concentration of amino acids in root exudates of different resistant cotton cultivars. They showed that 10 amino acids were detected in susceptible cultivars and the composition and amount of each amino acid were higher than resistant cultivars, in which only five amino acids were detected. Wu *et al.*(17) studied the effects of cotton root exudates on growth and development of *Verticillium dahlia*. Their results showed that the composition and amount of each amino acid in susceptible cultivars were significantly higher than resistant cultivars. Our results differed from Yuan *et al.*(20) and Wu *et al.*(17), possibly reflecting the different crops under study. Otherwise, the amount of each amino acid in resistant cultivars was high and lower in susceptible cultivars, which was consistent with studies of Yuan *et al.*(20) and Wu *et al.*(17). In addition, the composition of amino acids in root exudates from the two rootstocks (calabash and pumpkin) showed no significant differences, whereas, that of resistant and susceptible watermelon cultivars grafted onto the rootstocks did differ significantly from ungrafted seedlings. The amounts of amino acids in root exudates from seedlings of a resistant watermelon cultivar grafted onto calabash rootstock was significantly higher than ungrafted seedlings, whereas those in seedlings of a susceptible cultivar grafted onto pumpkin rootstock were significantly lower than in ungrafted seedlings. This might be evidence that amino acid concentrations in root exudates are related to resistance of watermelon to fusarium wilt. Grafting could enhance the resistance of watermelon to fusarium wilt, through the concentration of amino acids in root exudates, but this requires further investigation. Furthermore aspartic acid, glumatic acid, methionine, leucine and phenylalanine promoted the growth and development of *Fusarium oxysporum* f.sp. *nevium* at concentrations of 1.2 g·L^{-1} or 1.8 g·L^{-1}, a finding that differed from Zhen *et al*.(21), who showed that aspartic acid and glumatic acid significantly inhibited the mycelial growth of strawberry pathogenic bacteria. However, our results were in agreement with two previous reports (12, 14). Wang (14) showed that threonine, proline, valine, methionine, tyrosine, phenylalanine and lysine stimulated the growth of eggplant verticillium wilt. Liu *et al*. (12) showed that alanin, aspartic acid, lysine, proline and isoleucine in cotton root exudates stimulated the growth of cotton wilt pathogenic fusarium, but arignine, glutamic acid, lysine and serine had an inhibitory effect. In the present study, 0.6 g·L^{-1} methionine and phenylalanine inhibited the growth and development of *Fusarium oxysporum* f.sp. *nevium*. This illustrates that lower concentrations of amino acids can inhibit the growth and development of *Fusarium oxysporum* f.sp. *nevium*. The possible reasons for this need further examination. The differences in results between published studies may be reflecting the different pathogens investigated and the use of a single index of growth and development in the above studies.

Consequently, through selection for regulation of composition and concentration of amino acids in root exudates, resistance of watermelon to fusarium wilt could be improved in breeding programmes. This would be beneficial for the further study of autointoxication in watermelon and its relationship with the problem of continuous cropping.

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