

# Amino acid changes during the early stages of tomato wilt disease (*Verticillium albo-atrum*)

GEOFFREY RICHARD DIXON\*

School of Agriculture, Policy and Development, Earley Gate, Whiteknights Campus,  
University of Reading, Reading, United Kingdom

\*Corresponding author: [geoffrdixon@gmail.com](mailto:geoffrdixon@gmail.com)

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**Abstract:** Soil-borne pathogens such as *Verticillium* species, invade into the roots of many herbaceous and woody hosts. The xylem environment supplies these pathogens with a continuous flow of nitrogen-rich nutrition. Detailed quantitative increases in amino acids in the stems, petioles, leaflets and roots of young tomato plants infected with *Verticillium albo-atrum* the causal agent of wilt disease, are described in this paper for the first time. Results focus in particular on the vascular environment prior to the emergence of visual symptoms. Total amino acid concentrations in infected stems and petioles increased substantially at 144 and 216 h after inoculation. This effect was evident in leaflets at 216 h after inoculation. By 216 h most amino acid concentrations were substantially increased in stems, petioles and leaflets of infected plants relative to healthy controls. Earlier at 144 h in stems substantial increases were recorded for aspartic acid, threonine, serine, glutamic acid, glycine and ethanolamine. A similar picture emerged for petioles with the addition of increases in proline but not glycine. Amino acids increasing substantially in infected leaflets at 216 h were aspartic acid, glutamic acid and ethanolamine. In the infected roots there was relatively little difference in amino acid concentrations relative to healthy controls with the particular exceptions of proline and ethanolamine. By 18 days (432h), when visual symptoms were well advanced marked increases in amino acid concentrations were found for threonine, serine,  $\alpha$ -alanine, valine, methionine, *iso*-leucine, leucine, tyrosine, ethanolamine, ornithine, lysine, histidine and arginine.

**Keywords:** vascular pathogen; nitrogen; stress; syndrome; pre-visual symptoms

*Verticillium* wilt is an aggressive vascular disease, devastating many economically important herbaceous and woody crop hosts worldwide (Pegg & Brady 2002; Klosterman et al. 2009). Externally visible symptoms caused by the pathogen, *Verticillium albo-atrum*, to major valuable hosts such as tomato, commence with wilting, dwarfing, epinasty and leaf chlorosis followed by foliar necrosis and eventual host death. Internal colonisation results from spores and mycelial fragments moving upwards through the xylem this elicits host responses involving the formation of tyloses, gums and resultant browning of the vessels (Koike et al. 2007). Species of bacteria and fungi that have evolved lifestyle strategies as vascular pathogens exploit the continual

flow of nutrients within the xylem for growth and reproduction as recently reviewed for *Clavibacter michiganensis*, bacterial canker of tomato by Peritore-Galve et al. (2020). Earlier, Lowe-Powell et al. (2018a, b) working with *Ralstonia solanacearum*, another cause of bacterial wilt, highlighted the importance of understanding the nutritional environment surrounding vascular pathogens before and after infection and invasion. *In vitro* studies of *Verticillium dahliae* demonstrated that mycelial growth and the development of conidia are enhanced by supplies of amino acids (Duncan & Himelick 1987). The availability of amino acids at the root surface for the tomato root-colonizing bacterium *Pseudomonas fluorescens* was for example, described

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by Simons (1997). Stimulatory compounds included aspartic acid, glutamic acid, *iso*-leucine, leucine and lysine. Studies of root exudates of cotton cultivars found that those susceptible to disease caused by *V. dahlia*, contained greater concentrations of amino acids compared with resistant hosts (Jiang et al. 2005). The disruption of host nitrogen metabolism which releases amino-nitrogen compounds benefiting the pathogen is likely therefore, to be an early and important stage in pathogenesis. Despite this, information on the internal environment in xylem vessels during the pre-symptomatic stages of vascular pathogenesis is still very limited.

The objectives of the research reported here were twofold: examining the colonisation processes and anatomical host responses and in detail analysing accompanying changes in the amino-nitrogen environment of the xylem. Detailed information of protein and non-protein amino acids which were present in the early stages of colonisation up to, and including initial visual symptom expression is presented here. This data amplifies results from the analyses of vascular sap of extracted from tomatoes infected with *V. albo-atrum* (Dixon & Pegg 1972). That research identified increased concentrations of aspartic and glutamic acids, threonine, serine, proline, tyrosine, ornithine, lysine, histidine, and phenylalanine in diseased plants relative to extracts from healthy plants.

## MATERIAL AND METHODS

Cultures of *V. albo-atrum* and inoculum production followed the techniques described by Pegg and Brady (2002). This resulted in a comminuted mixture of conidia, mycelial fragments and bud cells with a concentration of  $10^7$  propagules/mL. Batches of diseased and healthy (control) tomato plants were grown and infected following the protocol described by Dixon (1971a). In the first series of experiments tissue samples were harvested at 12.00 h after 5 h illumination at 0, 24 (1), 72 (3), 144 (6) and 216 h (9 days) after inoculation. For a second series tissue samples were harvested 432 h (18 days) after inoculation. All tissue samples were prepared for analysis using methods as described by Dixon (1971a). An automatic amino acid analyser (Technicon 1965, SEAL Analytical Ltd., UK) produced 21-hour elutograms of amino acids using a sodium citrate buffer gradient and ninhydrin-hydrindantin colour reagent following methodologies developed

by the Company. The accuracy and repeatability of analyses produced by this machine were rigorously tested and established prior to the research reported here (Dixon 1971b).

## RESULTS

**Individual amino acids.** Early symptoms of epinasty and mild foliar chlorosis became visible in infected plants at 216 h samples, hence most of the amino acid changes observed in these experiments happened before these events. The effects of infection with *V. albo-atrum* on amino acid concentrations were most clearly identified in analyses of stem tissue (Table 1). Compared with values obtained at the time of inoculation (0 h) amino acid concentrations increased in diseased stems at 144 and 216 hours. Concentrations in the control stem tissue rose at the 24 h and subsequently declined. Amino acids increasing most consistently in diseased stems particularly at 144 and 216 h were aspartic acid, proline threonine, serine, glutamic acid, valine, *iso*-leucine and leucine. Proline increased most markedly in the 216 h sample. Amino acids such as aspartic acid, threonine, serine, glycine,  $\alpha$ -alanine, valine, *iso*-leucine, ornithine, lysine, histidine and arginine showed relatively little change between 0 and 144 h in the controls.

Similar trends were recorded for petiole tissue (Table 2). Concentrations in diseased petioles of glutamic acid, threonine, aspartic acid, proline, serine and non-protein ethanolamine increased substantially compared with healthy controls in the 144 and 216 h samples. Increases were recorded at 216 h for  $\alpha$ -alanine, leucine, *iso*-leucine, valine, glycine, histidine, lysine and phenylalanine. Values recorded for control petioles remained close to those measured at 0 h throughout the experiment with the exception of ethanolamine.

In the diseased leaflets (Table 3), ethanolamine concentration increased rapidly from 24 h and continued this trend at 144 and 216 hours. Serine and  $\alpha$ -alanine increased substantially by 216 h. Aspartic acid with the exception of the 72 hr sample, increased across the experimental period. Leucine, *iso*-leucine, valine, arginine and lysine each showed increased concentrations at 216 h compared with healthy controls. Concentrations of tyrosine, leucine, *iso*-leucine, valine, arginine, histidine lysine and  $\alpha$ -alanine in control leaflets remained similar to the 0 h values throughout the experiment.

Table 1. Free amino acid concentrations\* in cv. *Potentate* tomato plant stems 0, 24, 72, 144 and 216 hours after inoculation with *Verticillium albo-atrum*

| Amino acid     | Time after inoculation (hours) |          |          |          |          |          |          |          |          |
|----------------|--------------------------------|----------|----------|----------|----------|----------|----------|----------|----------|
|                | 0                              | 24       |          | 72       |          | 144      |          | 216      |          |
|                |                                | D        | H        | D        | H        | D        | H        | D        | H        |
| Aspartic acid  | 281.77                         | 354.62   | 348.47   | 271.77   | 313.76   | 486.05   | 371.78   | 534.46   | 267.15   |
| Threonine      | 110.97                         | 344.56   | 299.13   | 220.65   | 217.44   | 585.14   | 184.64   | 751.5    | 141.3    |
| Serine         | 159.72                         | 331.31   | 273.99   | 170.45   | 150.44   | 604.07   | 185.71   | 729.54   | 175.18   |
| Glutamic acid  | 560.68                         | 686.22   | 711.17   | 465.8    | 511.72   | 965.64   | 504.17   | 1 047.18 | 478.46   |
| Proline        | 37.24                          | 30.16    | 27.22    | 50.84    | 33.02    | 140.93   | 95.22    | 7 84.77  | 60.58    |
| Citrulline     | 18.42                          | 30.37    | 31.84    | 21.52    | 30.05    | 58.61    | 45.66    | 36.46    | 26.89    |
| Glycine        | 35.48                          | 84.55    | 70.09    | 54.6     | 51.42    | 123.35   | 50.27    | 106.46   | 34.24    |
| α-alanine      | 83.71                          | 124.17   | 134.96   | 106.07   | 86.7     | 178.92   | 88.37    | 179      | 85.64    |
| Valine         | 38.67                          | 101.7    | 97.47    | 55.5     | 43.6     | 74.59    | 45.32    | 138.97   | 44.73    |
| Methionine     | ni                             | ni       | ni       | ni       | ni       | ni       | ni       | ni       | ni       |
| Iso-leucine    | 26.42                          | 70.16    | 65.11    | 40.68    | 28.55    | 62.32    | 30.32    | 90.88    | 34.4     |
| Leucine        | 53.56                          | 68.74    | 81.71    | 76.35    | 54.67    | 99.99    | 64.51    | 136.65   | 72.6     |
| Tyrosine       | 14.43                          | 53.83    | 36.3     | 21.18    | 11.56    | 21.46    | 19.01    | 53.11    | 20.63    |
| Phenyl-alanine | 0                              | 64.46    | 50.5     | 46.07    | 39.98    | 0        | 27.22    | ni       | 45.96    |
| Ethanolamine   | 727.16                         | 816.89   | 528      | 488.26   | 652.08   | 1617     | 1192.8   | ni       | 1 113.9  |
| Ornithine      | 17.6                           | 30.7     | 32.73    | 35.83    | 33.62    | 35.5     | 27.21    | 46.8     | 20.86    |
| Lysine         | 59.2                           | 87.68    | 78.49    | 122.5    | 63.72    | 99.05    | 48.64    | 116.41   | 68.79    |
| Histidine      | 19.92                          | 41.61    | 41.06    | 26.34    | 32.22    | 63.6     | 33.44    | 91.41    | 25.23    |
| Arginine       | 37.73                          | 57.18    | 60.69    | 44.45    | 42.61    | 54.23    | 44.24    | 59.84    | 31.33    |
| Totals         | 2 282.68                       | 3 378.91 | 2 968.93 | 2 318.86 | 2 397.16 | 5 270.45 | 3 058.53 | 4903.44  | 2 747.87 |

\*amino acids listed according to the elution sequence beginning with the most acidic compounds; D – diseased; H – healthy; ni – peak could not be integrated; values are expressed as  $\mu\text{mole/kgm}$  fresh weight

Over the whole experimental period concentrations of most amino acids were relatively similar to the 0 h values in both infected and control roots (Table 4), with the exception of proline, serine, threonine, phenylalanine, glutamic acid and ethanolamine. Proline concentrations in diseased roots were considerably larger than those of controls. Phenylalanine and glutamic acid concentrations in diseased roots increased above those of controls at 72 and 144 h, respectively. Otherwise, there were only very limited differences between concentrations measured for diseased and control roots.

Most amino acids increased in infected leaves at 432 h after inoculation by when visual symptoms were well developed (Table 5). The large increase in free methionine, a sulphur containing amino acid of importance in protein bonding correlates with the appearance of visual symptoms when proteolysis would be well advanced. Conversion of aspartic acid, proline, phenylalanine and ethanolamine to neutral

and basic amino acids or to ninhydrin-negative substances, in diseased leaves may have been indicated by their decreased concentrations at 432 h. Absolute increases in amino acid concentrations in healthy cv. *Potentate* leaves at 18 days compared with 9 days may result from senescent leaves shunting nitrogen into younger, expanding leaves.

**Total amino acids.** Base line samples, 0 h, showed total amino acid concentrations were greatest in the leaflets, 5 301  $\mu\text{mole/kgm}$  fresh weight (Table 3) and lowest in the stems at 2 282  $\mu\text{mole/kgm}$  fresh weight (Table 1) with the petioles (Table 2) and roots (Table 4) containing 2 495 and 1 953  $\mu\text{mole/kgm}$  fresh weight, respectively. Analyses of total amino acids in both healthy and infected stems (Table 1) at 24 h after inoculation showed similar increases. Thereafter, in infected stems there were increases at 144 and 216 h in amino acid content relative to healthy controls. Total amino acid concentrations in infected petioles (Table 2) exceeded those in the

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Table 2. Free amino acid concentrations\* in cv. *Potentate* tomato plant petioles 0, 24, 72, 144 and 216 hours after inoculation with *Verticillium albo-atrum*

| Amino acid     | Time after inoculation (hours) |          |          |          |        |          |        |          |          |
|----------------|--------------------------------|----------|----------|----------|--------|----------|--------|----------|----------|
|                | 0                              | 24       |          | 72       |        | 144      |        | 216      |          |
|                |                                | D        | H        | D        | H      | D        | H      | D        | H        |
| Aspartic acid  | 314.43                         | 370.39   | 357.96   | 308.64   | 383.66 | 394.87   | 286.54 | 408.16   | 304.98   |
| Threonine      | 188.47                         | 253.19   | 238.33   | 187.51   | 209.35 | 320.07   | 196.18 | 467.23   | 140.48   |
| Serine         | 230.04                         | 401.4    | 276.6    | 197.15   | 226.08 | 436.8    | 217.6  | 720.89   | 217.66   |
| Glutamic acid  | 645.89                         | 780.66   | 669.4    | 585.16   | 659.58 | 915.06   | 663.12 | 817.73   | 612.39   |
| Proline        | 33.51                          | 51.01    | 50.5     | 51.13    | 46     | 171.87   | 78.05  | 697.6    | 68.04    |
| Citrulline     | 41.83                          | 26.38    | 20.69    | 23.09    | 26.61  | 42.21    | 35.85  | 22.89    | 23.06    |
| Glycine        | 44.13                          | 87.08    | 49.62    | 50.44    | 56.17  | 68.14    | 74.16  | 121.95   | 61.55    |
| α-alanine      | 116.67                         | 179.36   | 186.49   | 144.51   | 163.21 | 200.19   | 180.45 | 260.62   | 130.55   |
| Valine         | 36.46                          | 81.66    | 71.63    | 56.66    | 54.61  | 77.14    | 36.84  | 145.19   | 50.93    |
| Methionine     | 0                              | 0        | 0        | 0        | 0      | 0        | 0      | 0        | 0        |
| Iso-leucine    | 24.34                          | 46.63    | 42.8     | 34.78    | 34.8   | 44.39    | 27.49  | 78.3     | 26.9     |
| Leucine        | 42.82                          | 73.93    | 59.98    | 65.26    | 65.41  | 73.05    | 62.05  | 136.54   | 66.22    |
| Tyrosine       | 22.26                          | 51.56    | 36.93    | 21.97    | 21.51  | 38.95    | 24.21  | 55.74    | 22.03    |
| Phenyl-alanine | 21.29                          | 44.04    | 38.07    | 32.67    | 28.03  | 53.99    | 0      | 322.64   | 30.72    |
| Ethanolamine   | 595.72                         | 766.87   | 378.7    | 636.53   | 1 167  | 1292.6   | 729.3  | 2 448    | 689.56   |
| Ornithine      | 48.94                          | 44.98    | 42.98    | 30.66    | 37.64  | 8.79     | 15.18  | 63.97    | 30.48    |
| Lysine         | 46.29                          | 88.11    | 91.13    | 61.76    | 75.56  | 86.49    | 62.58  | 128.79   | 64.25    |
| Histidine      | 21.96                          | 21.92    | 20.06    | 17.37    | 21.53  | 24.73    | 15.24  | 60.73    | 21.46    |
| Arginine       | 20.32                          | 48.66    | 58.19    | 54.76    | 43.25  | 48.02    | 32.16  | 52.35    | 29.1     |
| Totals         | 2 495.37                       | 3 417.83 | 2 690.06 | 2 560.05 | 3 320  | 4 297.36 | 2 737  | 7 009.32 | 2 590.36 |

\*amino acids listed according to the elution sequence beginning with the most acidic compounds; D – diseased; H – healthy; values are expressed as μmole/kgm fresh weight

controls at 24 h by *circa* 700 μmole/kgm fresh weight. Resembling responses in the stems, concentrations in infected petioles and leaflets increased rapidly at 144 and 216 hours. Analyses of infected and healthy roots (Table 4) showed smaller differences in total amino acid concentrations until 216 hours. By 432 h total amino acid concentration in diseased leaves exceeded that of healthy plants by 166% (Table 5). The pathogen, *V. albo-atrum*, substantially colonised stems and petioles from 72 h onwards (Pegg & Brady 2002) an effect which closely aligned with the changes in nitrogen metabolism.

## DISCUSSION

Each of the 20 most common amino acids have unique roles as precursors to and constituents of proteins, and in plant development and metabolism (Nelson 2017). The methods used in this research allowed the resolution of 16 protein-forming

amino acids and three non-protein compounds, citrulline, ethanolamine and ornithine. Citrulline and ornithine are metabolically closely related and involved in plant growth. Ethanolamine is a building block for plant membrane phospholipids and signalling molecule (Courtinho et al. 2018) and is formed by decarboxylation of serine (Rontein et al. 2001). When plants experience stresses such as diseases, they accumulate amino acids (Sarhan et al 1982; Raitilack et al. 2019). An understanding of *Verticillium* spp. affecting host metabolism was reported by Pegg and Brady (2002). Nowhere, however, do they describe changes in individual amino acids from infection before and following visible symptoms expression. The research reported here helps redress that deficiency.

Analyses of amino acid concentrations in infected and healthy stems and petioles at 24 h rose substantially and then declined, possibly this indicates an oxidative burst resulting from root inoculation with

Table 3. Free amino acid concentrations\* in cv. *Potentate* tomato plant leaflets 0, 24, 72, 144 and 216 hours after inoculation with *Verticillium albo-atrum*

| Amino acid     | Time after inoculation (hours) |          |          |          |          |          |          |          |          |
|----------------|--------------------------------|----------|----------|----------|----------|----------|----------|----------|----------|
|                | 0                              | 24       |          | 72       |          | 144      |          | 216      |          |
|                |                                | D        | H        | D        | H        | D        | H        | D        | H        |
| Aspartic acid  | 920.87                         | 1 217.24 | 918.77   | 1 142.78 | 1 142.5  | 1 258.75 | 1 141    | 1 411.35 | 1 236.4  |
| Threonine      | 364.93                         | 366.77   | 351.16   | 472.97   | 495.32   | 480.71   | 773.62   | 444.8    | 496.16   |
| Serine         | 464.43                         | 552.31   | 421.03   | 276.52   | 371.98   | 606.32   | 619.07   | 822.91   | 397.79   |
| Glutamic acid  | 1 338.6                        | 845.07   | 834.56   | 720.86   | 708.84   | 1 205.55 | 1 011.3  | 674.46   | 502.73   |
| Proline        | 147.11                         | 115.96   | 139.11   | 282.47   | 138.5    | 257.24   | 270.94   | 729.19   | 330.05   |
| Citrulline     | ni                             | ni       | ni       | ni       | ni       | 101.22   | 122.88   | 42.56    | ni       |
| Glycine        | 150.04                         | 184.89   | 199.82   | 229.36   | 215.7    | 152.27   | 246.94   | 215.55   | 188.08   |
| α-alanine      | 630.62                         | 593.99   | 629.3    | 570.51   | 639.36   | 877.74   | 935.17   | 1 155.25 | 727.35   |
| Valine         | 104.53                         | 169.62   | 171.02   | 102.22   | 115.4    | 157.09   | 139.67   | 222.16   | 135.5    |
| Methionine     | ni                             | ni       | ni       | ni       | ni       | 49.47    | 54.44    | 43.82    | 24.45    |
| Iso-leucine    | 66.6                           | 92.7     | 92.39    | 81.21    | 69.44    | 87.19    | 80.25    | 129.71   | 74.93    |
| Leucine        | 114.36                         | 187.35   | 191.29   | 161.49   | 135.64   | 182.69   | 172.64   | 283.88   | 148.96   |
| Tyrosine       | 36.29                          | 129.2    | 61.02    | 91.71    | 58.82    | 75.22    | 53.47    | 120.46   | 72.59    |
| Phenyl-alanine | 93.27                          | 266.77   | 126.17   | 219.21   | 94.17    | 117.7    | 96.24    | 217.37   | 125.47   |
| Ethanolamine   | 681.08                         | 726.99   | 617.23   | 1 260    | 530.65   | 1 436.2  | 731.46   | ni       | ni       |
| Ornithine      | ni                             | ni       | ni       | ni       | ni       | ni       | ni       | 58.46    | 105.3    |
| Lysine         | 101.22                         | 157.38   | 173.66   | 121.7    | 122.83   | 156.11   | 138.43   | 213.27   | 124.12   |
| Histidine      | 25.71                          | 55.74    | 75.64    | 42.99    | 31.33    | 46.8     | 28.69    | 70.01    | 51.52    |
| Arginine       | 61.54                          | 76.03    | 86.81    | 78.15    | 66.53    | 82.39    | 68.21    | 115      | 58.37    |
| Totals         | 5 301.2                        | 5 738.01 | 5 088.98 | 5 854.15 | 4 937.01 | 7 330.66 | 6 684.42 | 6 970.21 | 4 799.77 |

\*amino acids listed according to the elution sequence beginning with the most acidic compounds; D – diseased; H – healthy; ni – peak could not be integrated; values are expressed as μmole/kgm fresh weight

live and killed spores. Infection by *V. albo-atrum* subsequently disrupted host amino acid metabolism from 72 h after inoculation well in advance of visual symptom expression. Increases in amino acids concentrations were largest in stems reflecting where pathogen colonisation is greatest compared with petioles and leaflets (Pegg & Brady 2002). The pathogen, itself, is unlikely to contribute towards the amino compounds in the xylem (Dixon & Pegg 1972). But as demonstrated by Duncan and Himelick (1987) *in vitro* pathogen growth and sporulation are stimulated by the presence of amino acids. *In planta* research reported here shows that pathogenesis caused by *V. albo-atrum* mobilizes significant host derived amino acids.

Amino acids increased in stems, petioles and leaflets up to 216 h after inoculation by which time early, mild visual symptoms had appeared. All amino acids increased in diseased leaves, including petioles, at 432 hours. Concentrations of proline, phe-

nylalanine and ethanolamine were, however, higher at 216 than at 432 hours. This might reflect phenylalanine contributing to enhanced flavanoid production as part of symptom expression involving chlorosis at 216 hours. Excess ammonia resulting from ethanolamine at 216 h could have induced the subsequent severe foliar chlorosis and necrosis seen at 432 hours. Increased ethanolamine might also indicate disruption of host lipid metabolism. Lipid metabolism in plants affected by vascular pathogens has received very little attention.

Concentrations in infected and healthy roots were similar with the exceptions of proline and ethanolamine. Rising proline concentrations are indicative of plants experiencing both abiotic and biotic stresses inducing antioxidant defensive events and as a signaling molecule (Verbruggen & Hermans 2008; Qaiser et al. 2012). In the current research increased proline in the petioles and leaflets and to a lesser extent in stems was typical



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Table 4. Free amino acid concentrations\* in cv. *Potentate* tomato plant roots 0, 24, 72, 144 and 216 hours after inoculation with *Verticillium albo-atrum*

| Amino acid     | Time after inoculation (hours) |          |          |          |         |          |          |          |          |
|----------------|--------------------------------|----------|----------|----------|---------|----------|----------|----------|----------|
|                | 0                              | 24       |          | 72       |         | 144      |          | 216      |          |
|                |                                | D        | H        | D        | H       | D        | H        | D        | H        |
| Aspartic acid  | 209.83                         | 166.26   | 145.04   | 179.79   | 189.37  | 166.84   | 246.64   | 214.64   | 207.42   |
| Threonine      | 109.17                         | 241.06   | 257.89   | 239.07   | 180.71  | 210.48   | 221      | 207.87   | 158.28   |
| Serine         | 130.81                         | 149.63   | 173.45   | 187.03   | 120.64  | 231.16   | 173.54   | 204.15   | 191.26   |
| Glutamic acid  | 410.83                         | 510.73   | 469.34   | 821.37   | 642.38  | 832.9    | 773.42   | 611.08   | 674.69   |
| Proline        | 29.38                          | 28.53    | 32.63    | 66.29    | 60.38   | 163.14   | 123.86   | 140.57   | 69.01    |
| Citrulline     | 10.5                           | 12.65    | ni       | 13.44    | 12.25   | ni       | 16.88    | ni       | ni       |
| Glycine        | 69.19                          | 85.14    | 104.26   | 111.47   | 56.92   | 78.33    | 93.55    | 41.46    | 86.64    |
| α-alanine      | 61.59                          | 91.1     | 68.05    | 110.07   | 116.64  | 71.66    | 74.02    | 78.98    | 92.91    |
| Valine         | 41                             | 78.65    | 73.53    | 77.68    | 61.75   | 86.48    | 67.26    | 77.11    | 88.51    |
| Methionine     | ni                             | ni       | ni       | ni       | ni      | ni       | ni       | ni       | ni       |
| Iso-leucine    | 31.86                          | 52       | 58.3     | 49.92    | 44.67   | 61.37    | 49.18    | 61.96    | 46.47    |
| Leucine        | 73.03                          | 99.25    | 91.41    | 113.75   | 79.21   | 120.53   | 104.83   | 99.56    | 110.66   |
| Tyrosine       | 49.97                          | 10.31    | 13.05    | 11.1     | 6.64    | 14.11    | 11.41    | 5.34     | 4.85     |
| Phenyl-alanine | 30.7                           | 39.5     | 70.28    | 61.93    | 45.17   | 193.59   | 47.98    | 37.41    | 71.01    |
| Ethanolamine   | 537.56                         | 446.65   | 297.75   | 809.13   | 784.9   | 389.44   | 632.17   | 1 761.5  | 890.36   |
| Ornithine      | 35.49                          | 41.37    | 42.41    | 37.4     | 57.07   | ni       | 28.14    | 62.17    | 37.58    |
| Lysine         | 43.66                          | 70.32    | 50.4     | 83.29    | 48.17   | 107.23   | 72.83    | 75.58    | 87.16    |
| Histidine      | 33.34                          | 53.55    | 53.53    | 58.93    | 39.47   | 66.41    | 47.45    | 56.45    | 62.17    |
| Arginine       | 45.24                          | 71.37    | 61.1     | 81.1     | 57.76   | 68.15    | 54.51    | 53.81    | 107.14   |
| Totals         | 1 953.15                       | 2 248.07 | 2 062.42 | 3 112.76 | 2 604.1 | 2 861.82 | 2 838.67 | 3 789.64 | 2 986.12 |

\*amino acids listed according to the elution sequence beginning with the most acidic compounds; D – diseased; H – healthy; ni – peak could not be integrated; values are expressed as μmole/kgm fresh weight

of pre-symptomatic diseased plants. Wilt disease induces epinasty of petioles and chlorosis of leaflets and the early presence of proline is a metabolic signal indicative of increasing distress. Additionally, despite cv. *Potentate* being classed as susceptible to *V. albo-atrum* the presence of proline might also suggest the activation of a form of generalised resistance response. This could be the case for aspartic acid, valine, and glycine which have been ascribed roles in resistance to *Verticillium* (NaNa et al. 2017). That suggestion might be supported by studies of leucine which is mobilised in defensive responses (Kawchuk 2010).

Aspartic and glutamic acids and serine are the basis of protein construction pathways (Zeier 2013) and sources of other amino acids by transamination. Considerable quantities were present in stems and petioles in early pathogenesis. The later appearance of increased concentrations of neutral and basic amino acids may indicate protein breakdown as visual symptoms developed. Disruption of tyrosine me-

tabolism could increase the loss of foliar chlorophyll with its pivotal role in photosynthesis (Nelson 2018).

This research established for the first time in detail that the disruption of amino acid metabolism is a pre-visual symptom of tomato wilt disease caused by *V. albo-atrum*. Increased concentrations of amino acids were identified within 72 h of inoculation, well before visual symptoms appearing by 216 hours. Disruption closely correlated with associated studies of the presence of the pathogen in xylem vessels particularly in the stems and leaflets. Compounds such as ethanolamine and proline are indicative of disease induced stress and the former contributing to epinasty, chlorosis and necrosis while the latter may suggest forms of resistance response possibly also associated with the presence of increased leucine. Initially both healthy and infected tomato seedlings exhibited increased amino acid concentrations, possibly indicating an oxidative burst event resulting from stresses caused by the presence of living and killed spores.

Table 5. Free amino acid content\* of cv. *Potentate* leaves 432 hours after inoculation with *Verticillium albo-atrum*

| Amino acid    | Healthy   | Diseased  |
|---------------|-----------|-----------|
| Aspartic acid | 1 610     | 1 424.8   |
| Threonine     | 668.58    | 1 925.3   |
| Serine        | 1 074     | 3 038     |
| Glutamic acid | 2 310.6   | 2 730.1   |
| Proline       | 5781.8    | 7 677.3   |
| Glycine       | 152.51    | 264.87    |
| α-alanine     | 864.98    | 2 113.2   |
| Valine        | 216.02    | 475.38    |
| Methionine    | 35.19     | 115.7     |
| Iso-leucine   | 122.96    | 264.11    |
| Leucine       | 236.43    | 617.66    |
| Tyrosine      | 60.95     | 116.65    |
| Phenylalanine | 153.25    | 175.86    |
| Ethanolamine  | 807.92    | 2 147     |
| Ornithine     | 117.33    | 284.96    |
| Lysine        | 202.73    | 500.07    |
| Histidine     | 61.68     | 176.53    |
| Arginine      | 93.25     | 228.46    |
| Totals        | 14 570.18 | 24 275.95 |

\*amino acids listed according to the elution sequence, the most acidic compounds first; citrulline could not be quantified; values are expressed as μmole/kgm fresh weight

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