EXTENDED MATERIALS AND METHODS

Chemical synthesis of VdAMP3

VdAMP3 was chemically synthesized and purified (≥ 95% purity) by GenScript (Piscataway, NJ, USA) using the PepPower™ platform. Lyophilized VdAMP3 was solubilized in ultrapure water (MQ) and stored at -20^{o}C until use.

Microbial isolates

Bacterial strains *B. subtilis* AC95, *S. xylosus* M3, *P. corrugata* C26, *Streptomyces* sp. NE-P-8 and *Ralstonia* sp. M21 were obtained from our in-house endophyte culture collection. Bacterial strains *Novosphingobium* sp. (NCCB 100261) and *Sphingobacterium canadese* (NCCB100125) were obtained from the Westerdijk Fungal Biodiversity Institute (Utrecht, the Netherlands). Fungal strains *Saccharomyces cerevisiae* H15 and *Trichoderma viride* were obtained from our in-house culture collection. Fungal strains *Cyberlindnera jadinii* (DSM 70167), *Cordyceps militaris* (DSM 1153), *Debaryomyces vanrijiae* (DSM 70252), *Meyerozyma amylolytica* (DSM 27310) and *Rhodotorula bogoriensis* (DSM 70872) were obtained from the Leibniz Institute DSMZ.

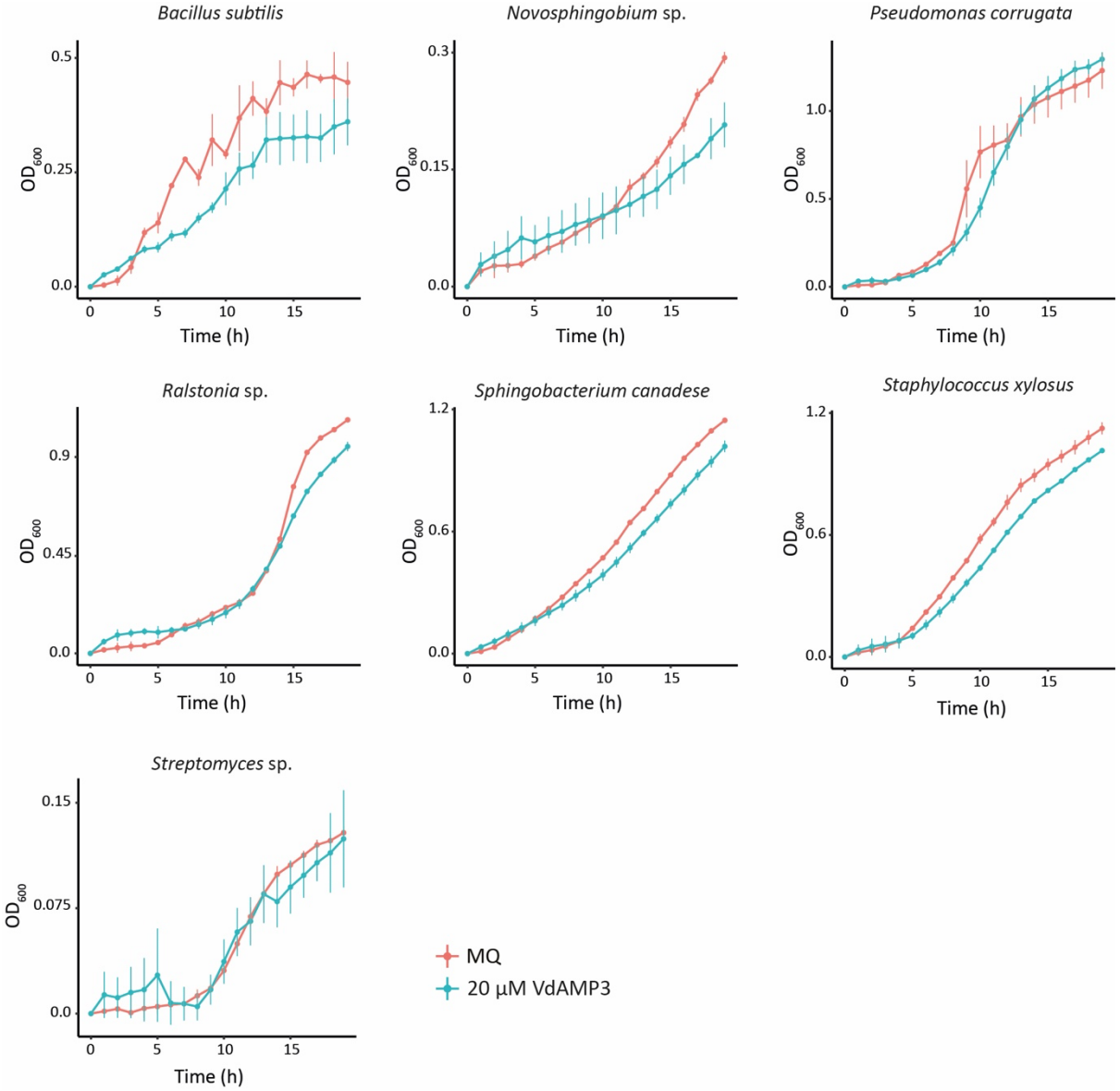
Inoculation assays

Three-week-old *N. benthamiana* seedlings grown in the greenhouse at 21^{o}C/19^{o}C during 16h/8h day/night periods, respectively, with 70% relative humidity, were inoculated with *V. dahliae* through root-dip inoculation as described previously (60). After 14 days, above-ground parts of the *N. benthamiana* plants were harvested and stored at -20^{o}C. Alternatively, above-ground parts were collected and transferred to plastic bags (volume = 500 mL) and incubated for four weeks at room temperature. Next, all *N. benthamiana* samples were ground using mortar and pestle. Subsequent genomic DNA isolation and *V. dahliae* biomass quantification was performed using the primers listed in Supplementary Table 3. Alternatively, to validate the integrity and activity of the microsclerotia formed by *V. dahliae* WT and the *VdAMP3* deletion mutant, we mixed the ground material of four

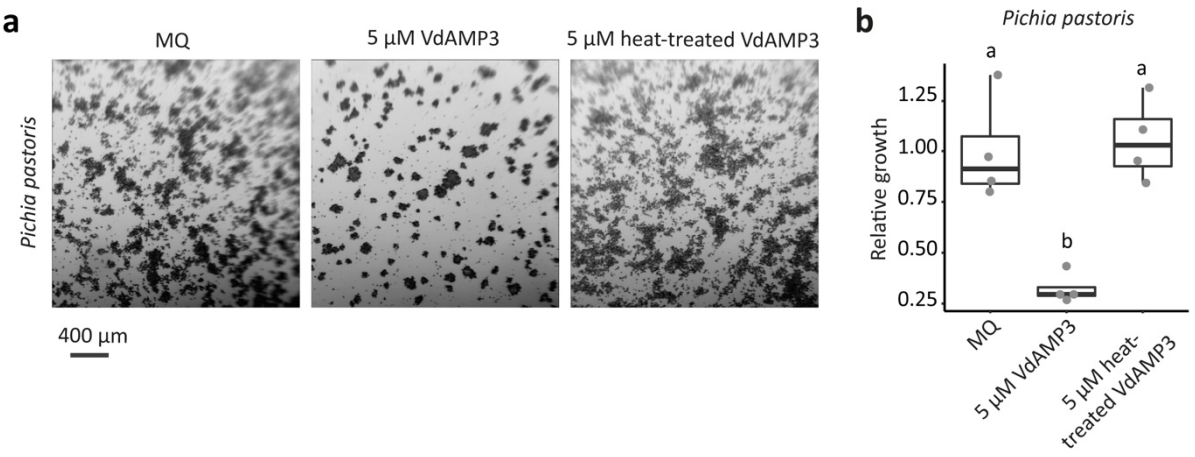
plants with 200 mL of potting soil and planted two-week-old *N. benthamiana* seedlings on this mixture. The phenotypes of the diseased *N. benthamiana* plants were imaged five weeks later.

Fluorescence microscopy

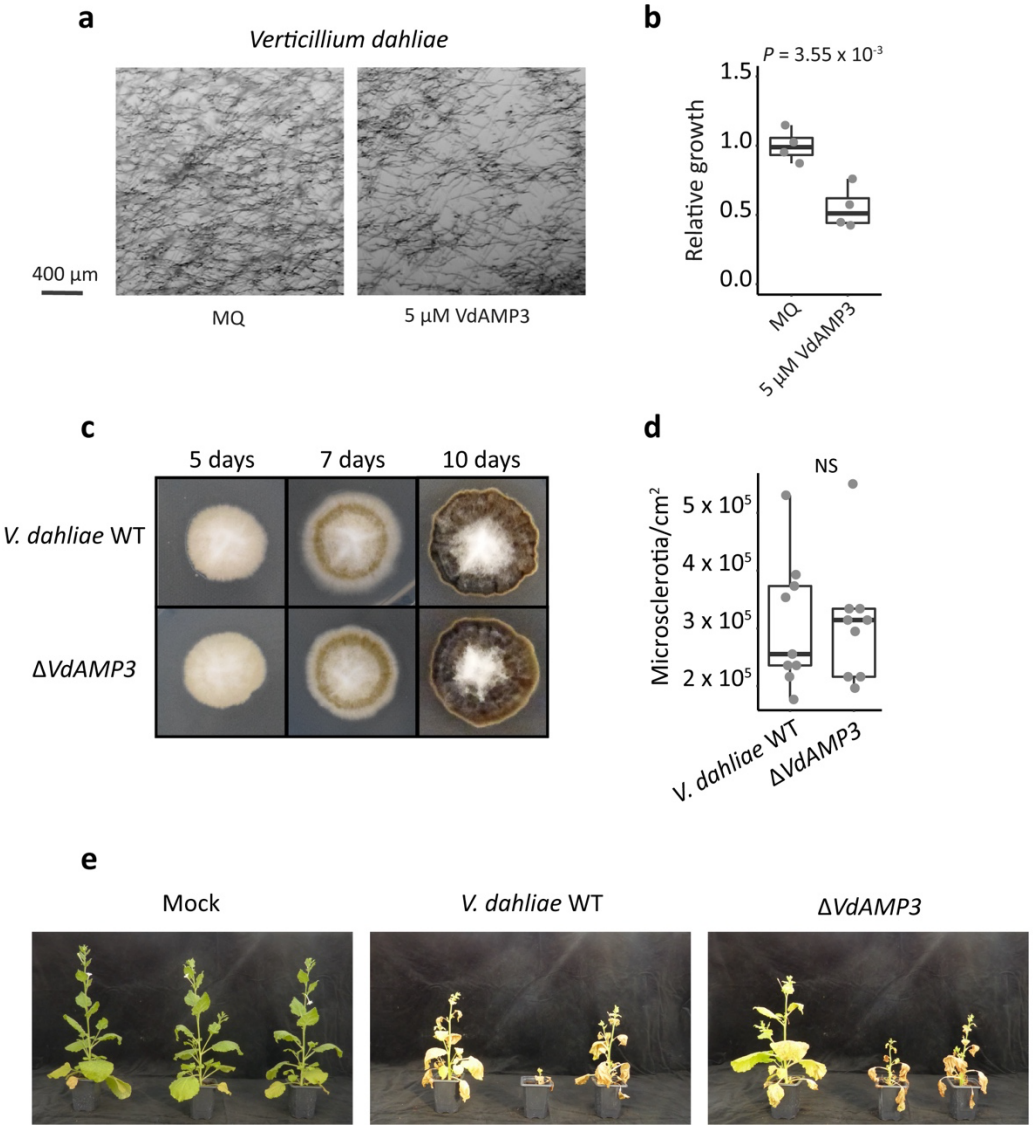
Conidiospores of the *pVdAMP3::eGFP* reporter strain were harvested from a PDA plate and diluted to a final concentration of 10^5 conidiospores/mL in 0.1x Czapek Dox medium. The suspension was incubated for one week at room temperature to allow hyphae to grow and microsclerotia to form. Finally, eGFP accumulating in the fungal cells was detected using a Nikon ECLIPSE 90i microscope.



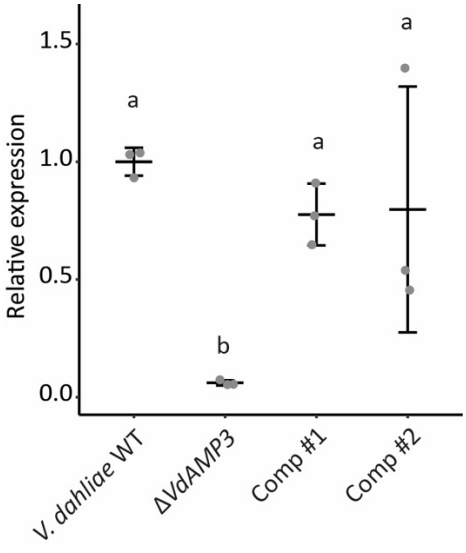
Supplementary Figure 1. VdAMP3 does not markedly impact bacterial growth. *In vitro* growth of plant-associated bacterial isolates in low salt LB is not, or only marginally, affected in the presence of 20 µM VdAMP3.



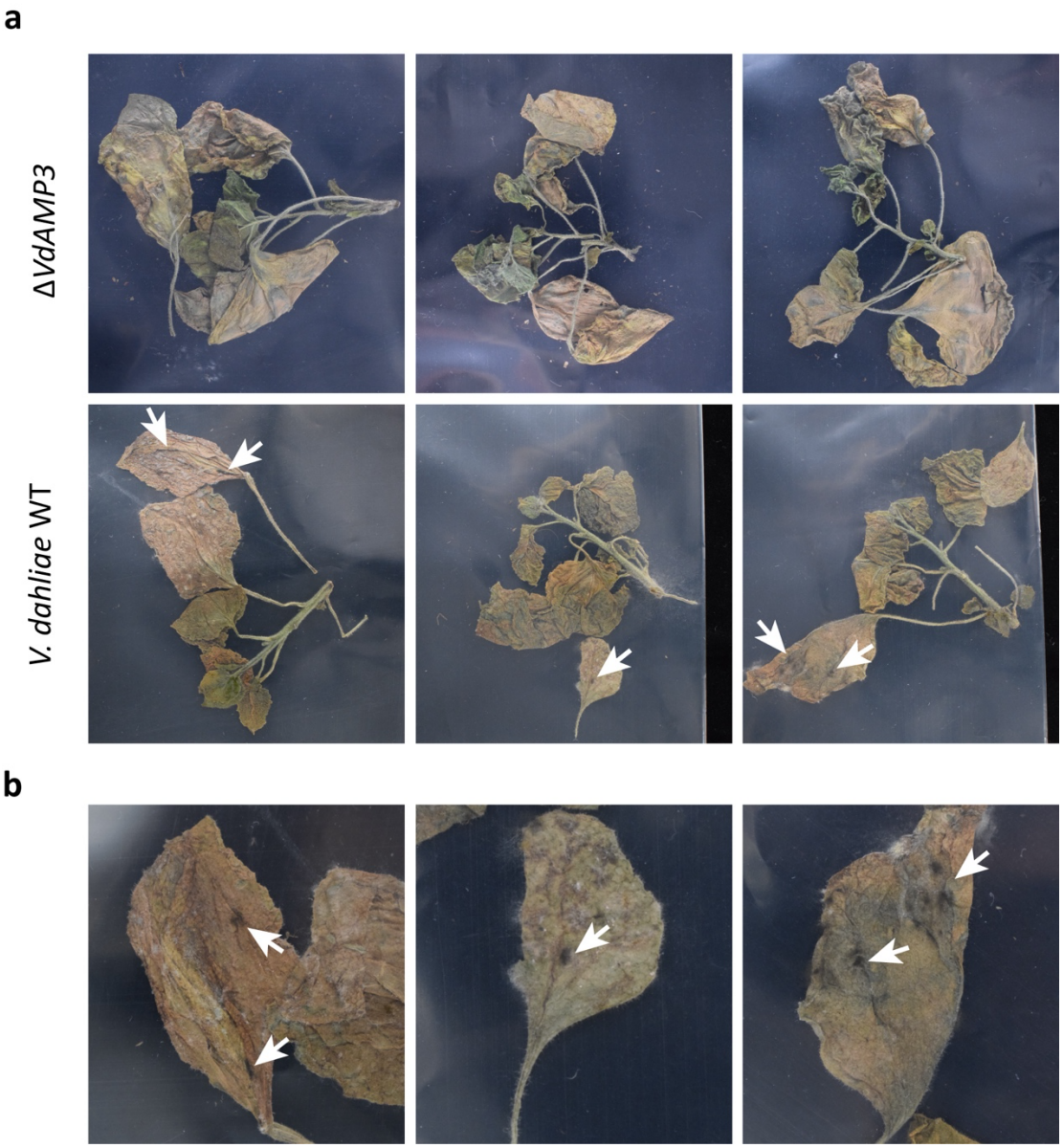
Supplementary Figure 2. Heat treatment abolishes antifungal activity of VdAMP3. Microscopic pictures of *Pichia pastoris* grown in 5% potato dextrose broth supplemented with ultrapure water (MQ), 5 µM VdAMP3 or 5 µM heat-treated VdAMP3. Pictures were taken after 48 hours of incubation. **(b)** Fungal growth as displayed in (**a**) was quantified using ImageJ (one-way ANOVA and Tukey's post-hoc test; $p<0.01$; N=4).



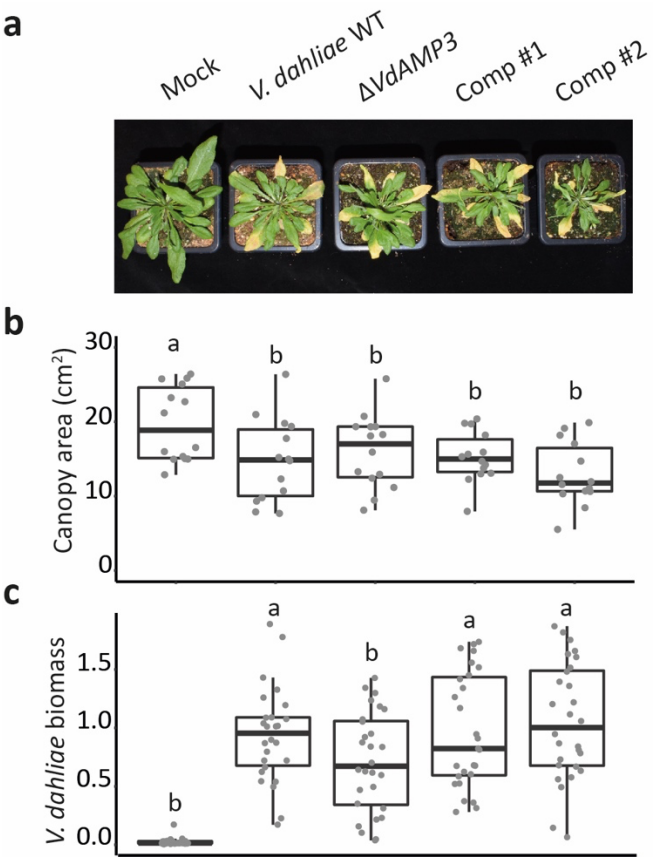
Supplementary Figure 3. *VdAMP3* does not impact *V. dahliae* growth and development. (a) Exogenously applied VdAMP3 impairs hyphal growth of *V. dahliae*. Microscopic pictures of *V. dahliae* grown in 5% potato dextrose broth supplemented with 5 µM VdAMP3 or ultrapure water (MQ), pictures were taken after 24 hours of incubation. **(b)** Growth as displayed in (**a**) was quantified using ImageJ (unpaired two-sided student's t-test; N=4). **(c-d)** Deletion of *VdAMP3* does not impact *V. dahliae* growth or microsclerotia formation *in vitro*. **(c)** Morphology of wild-type *V. dahliae* and the *VdAMP3* deletion mutant following five, seven and ten days of *in vitro* growth on PDA. **(d)** Deletion of *VdAMP3* does not impair microsclerotia formation. After ten days, the colonies as shown in **(c)** were processed and the number of microsclerotia per cm^2 was determined using a haemocytometer. No significant difference in microsclerotia formation was observed (unpaired student t-test N=9). **(e)** Deletion of *VdAMP3* does not impact the ability of microsclerotia to cause disease. Phenotypes of seven-week-old *N. benthamiana* plants grown on mock-treated potting soil or potting soil supplemented with microsclerotia of *V. dahliae* WT or the *VdAMP3* deletion mutant.



Supplementary Figure 4. Expression of *VdAMP3* in *V. dahliae* mutants. Expression of *VdAMP3* relative to *VdGAPDH* during microsclerotia formation by *V. dahliae* WT and the *VdAMP3* deletion and complementation mutants after seven days of cultivation in Czapek Dox medium. Letters represent statistically significant differences (one-way ANOVA and Tukey's post-hoc test; $p<0.05$; N=3).



Supplementary Figure 5. *VdAMP3* contributes to *V. dahliae* microsclerotia formation in decaying *N. benthamiana* phyllosphere. (a) Phenotypes of *N. benthamiana* plants colonized by *V. dahliae* WT or the *VdAMP3* deletion mutant that were harvested at 14 days post inoculation and subsequently incubated in plastic bags for 28 days. The white arrows indicate dark patches of mycelium with *V. dahliae* microsclerotia found on plants colonized by *V. dahliae* WT, but not by the *VdAMP3* deletion mutant. **(b)** Close-up of the *N. benthamiana* tissues harboring the patches with microsclerotia.



Supplementary Figure 6. VdAMP3 contributes to *V. dahliae* biomass accumulation in *Arabidopsis thaliana* but does not influence development of disease symptoms. (a) Deletion of *VdAMP3* does not affect establishment of Verticillium wilt disease in *A. thaliana*. Photos display representative phenotypes of *A. thaliana* plants 21 days post inoculation with *V. dahliae* WT and *VdAMP3* deletion and complementation mutants. **(b)** Canopy area of *A. thaliana* plants inoculated by the different *V. dahliae* genotypes. Different letter labels represent statistically significant differences when compared with *V. dahliae* WT (unpaired student's t-test; N=14). **(c)** Relative *V. dahliae* biomass in above-ground *A. thaliana* tissues determined with real-time PCR. Different letter labels represent statistically significant differences when compared with *V. dahliae* WT (unpaired student's t-test; N≥26).

Supplementary Table 1: DESeq2 output for the differentially abundant bacterial genera in the decaying phyllosphere of *N. benthamiana* plants colonized by *V. dahliae* wild type compared to the *VdAMP3* deletion mutant

Base mean	log2FC	lfcSE	stat	p value	p adjusted	Genus
6.57E+03	-6.60E+00	1.13E+00	-5.82E+00	5.88E-09	2.26E-06	*Advenella*
1.43E+01	-2.17E+01	3.91E+00	-5.54E+00	2.99E-08	5.76E-06	*Absicoccus*
8.83E+01	2.65E+00	6.25E-01	4.23E+00	2.31E-05	2.97E-03	*Azospirillum*
3.87E+01	2.97E+00	7.72E-01	3.85E+00	1.18E-04	6.00E-03	*Duganella*
8.74E+01	3.16E+00	8.36E-01	3.78E+00	1.56E-04	6.00E-03	*Chthoniobacter*
9.34E+03	2.76E+00	7.30E-01	3.78E+00	1.59E-04	6.00E-03	*Massilia*
3.16E+02	-3.09E+00	8.23E-01	-3.76E+00	1.71E-04	6.00E-03	*Ochrobactrum*
5.66E+01	-3.76E+00	1.08E+00	-3.47E+00	5.13E-04	1.52E-02	*Brenneria*
2.74E+01	2.24E+00	6.71E-01	3.34E+00	8.37E-04	2.12E-02	*Paenibacillus*
5.08E+01	-3.14E+00	9.45E-01	-3.33E+00	8.82E-04	2.12E-02	*Rouxiella*
1.32E+03	2.15E+00	6.62E-01	3.25E+00	1.15E-03	2.61E-02	*Caulobacter*
5.62E+02	2.59E+00	8.32E-01	3.11E+00	1.87E-03	3.99E-02	*Cellvibrio*
9.76E+02	2.46E+00	7.94E-01	3.09E+00	1.97E-03	3.99E-02	*Mucilaginibacter*
1.85E+01	-3.47E+00	1.13E+00	-3.08E+00	2.10E-03	4.04E-02	*Adhaeribacter*
7.36E+02	1.68E+00	5.55E-01	3.02E+00	2.50E-03	4.58E-02	*Flavobacterium*
6.39E+01	2.49E+00	8.28E-01	3.01E+00	2.63E-03	4.59E-02	*Taibaiella*
2.72E+03	2.34E+00	7.89E-01	2.97E+00	2.98E-03	4.59E-02	*Dyadobacter*
5.20E+01	-2.35E+00	7.98E-01	-2.94E+00	3.27E-03	4.84E-02	*Pusillimonas*
2.76E+03	1.43E+00	5.02E-01	2.84E+00	4.46E-03	6.13E-02	*Paraburkholderia*
1.21E+01	-2.97E+00	1.06E+00	-2.79E+00	5.25E-03	6.60E-02	*Rhizorhapis*
2.26E+01	-2.87E+00	1.08E+00	-2.65E+00	7.97E-03	8.98E-02	*Telmatospirillum*
4.21E+00	5.25E+00	1.98E+00	2.65E+00	8.03E-03	NA	*Myroides*
5.77E+03	-2.03E+00	7.67E-01	-2.65E+00	8.15E-03	8.98E-02	*Achromobacter*
1.02E+04	-1.59E+00	6.01E-01	-2.65E+00	8.16E-03	8.98E-02	*Sphingobium*
6.05E+00	4.00E+00	1.53E+00	2.61E+00	9.18E-03	9.80E-02	*Flavipsychrobacter*
7.67E+01	2.94E+00	1.13E+00	2.60E+00	9.42E-03	9.80E-02	*Archangium*
1.19E+04	-2.12E+00	8.21E-01	-2.58E+00	9.82E-03	9.89E-02	*Enterobacter*
1.29E+01	-2.64E+00	1.03E+00	-2.58E+00	1.00E-02	9.89E-02	*Peribacillus*
5.32E+01	2.79E+00	1.10E+00	2.53E+00	1.15E-02	1.08E-01	*Methylovorus*
2.90E+01	-1.92E+00	7.80E-01	-2.46E+00	1.40E-02	1.22E-01	*Candidimonas*
9.40E+01	-2.37E+00	1.01E+00	-2.35E+00	1.89E-02	1.58E-01	*Serratia*
1.54E+01	-2.60E+00	1.12E+00	-2.32E+00	2.04E-02	1.64E-01	*Leclercia*
7.76E+01	-2.00E+00	8.63E-01	-2.32E+00	2.04E-02	1.64E-01	*Rahnella*
8.42E+00	3.08E+00	1.37E+00	2.25E+00	2.46E-02	1.83E-01	*Gemmobacter*
4.26E+04	-2.44E+00	1.09E+00	-2.25E+00	2.48E-02	1.83E-01	*Pantoea*
7.68E+02	-1.68E+00	7.58E-01	-2.22E+00	2.63E-02	1.89E-01	*Bordetella*
2.47E+01	-2.43E+00	1.10E+00	-2.22E+00	2.65E-02	1.89E-01	*Tatumella*

5.52E+00	3.04E+00	1.39E+00	2.19E+00	2.87E-02	1.93E-01	*Thiomonas*
1.05E+01	2.34E+00	1.07E+00	2.19E+00	2.87E-02	1.93E-01	*Gilvimarinus*
3.93E+02	1.33E+00	6.08E-01	2.18E+00	2.91E-02	1.93E-01	*Ramlibacter*
3.53E+00	-5.46E+00	2.56E+00	-2.13E+00	3.31E-02	NA	*Listeria*
1.20E+02	-1.81E+00	8.69E-01	-2.08E+00	3.77E-02	2.34E-01	*Brevundimonas*
3.76E+02	1.47E+00	7.38E-01	1.99E+00	4.69E-02	2.77E-01	*Asticcacaulis*
7.80E+02	1.58E+00	7.95E-01	1.99E+00	4.71E-02	2.77E-01	*Shinella*
2.22E+01	1.84E+00	9.27E-01	1.98E+00	4.76E-02	2.77E-01	*Cytophaga*
9.32E+00	-1.88E+00	9.51E-01	-1.98E+00	4.83E-02	2.77E-01	*Porphyrobacter*

Supplementary Table 2: DESeq2 output for the differentially abundant fungal genera in the decaying phyllosphere of *N. benthamiana* plants colonized by *V. dahliae* wild type compared to the *VdAMP3* deletion mutant

Base mean	log2FC	lfcSE	stat	pvalue	padj	Genus
7.20E+01	-3.14E+00	7.70E-01	-4.08E+00	4.50E-05	3.87E-03	*Clavispora*
2.26E+01	-7.19E+00	1.77E+00	-4.05E+00	5.03E-05	3.87E-03	*Cordyceps*
1.55E+01	-5.85E+00	1.51E+00	-3.86E+00	1.11E-04	6.00E-03	*Trichomonascus*
2.64E+01	-5.10E+00	1.34E+00	-3.80E+00	1.43E-04	6.00E-03	*Akanthomyces*
9.44E+03	-4.13E+00	1.15E+00	-3.60E+00	3.19E-04	1.02E-02	*Fusarium*
7.01E+01	3.29E+00	9.81E-01	3.36E+00	7.91E-04	2.12E-02	*Terfezia*
2.22E+01	3.99E+00	1.34E+00	2.98E+00	2.84E-03	4.59E-02	*Rhodotorula*
1.82E+01	-2.76E+00	9.28E-01	-2.98E+00	2.92E-03	4.59E-02	*Beauveria*
4.87E+01	-8.30E+00	2.86E+00	-2.90E+00	3.71E-03	5.29E-02	*Escovopsis*
1.44E+02	-2.81E+00	9.97E-01	-2.81E+00	4.91E-03	6.51E-02	*Yamadazyma*
1.21E+01	3.51E+00	1.26E+00	2.79E+00	5.31E-03	6.60E-02	*Ascosphaera*
5.54E+03	-3.18E+00	1.19E+00	-2.68E+00	7.45E-03	8.96E-02	*Trichoderma*
6.49E+00	-3.79E+00	1.50E+00	-2.53E+00	1.14E-02	1.08E-01	*Xylaria*
5.53E+01	2.89E+00	1.16E+00	2.48E+00	1.32E-02	1.21E-01	*Golovinomyces*
1.88E+01	-2.17E+00	8.79E-01	-2.47E+00	1.36E-02	1.21E-01	*Valsa*
5.03E+00	-3.60E+00	1.48E+00	-2.44E+00	1.49E-02	1.27E-01	*Acremonium*
2.18E+00	-4.77E+00	2.04E+00	-2.34E+00	1.94E-02	NA	*Meyerozyma*
2.92E+00	-4.17E+00	1.79E+00	-2.33E+00	2.00E-02	NA	*Scedosporium*
4.94E+00	-3.36E+00	1.46E+00	-2.30E+00	2.14E-02	1.68E-01	*Ustilaginoidea*
2.98E+01	-2.60E+00	1.15E+00	-2.26E+00	2.35E-02	1.81E-01	*Debaryomyces*
6.66E+01	-1.45E+00	6.64E-01	-2.19E+00	2.86E-02	1.93E-01	*Phaeoacremonium*
1.58E+01	2.43E+00	1.15E+00	2.11E+00	3.46E-02	2.26E-01	*Morchella*
2.10E+01	-1.97E+00	9.38E-01	-2.11E+00	3.52E-02	2.26E-01	*Neonectria*
1.09E+01	-1.77E+00	8.45E-01	-2.10E+00	3.59E-02	2.26E-01	*Spathaspora*
5.98E+00	-3.07E+00	1.51E+00	-2.04E+00	4.12E-02	2.52E-01	*Pochonia*
2.02E+00	-4.67E+00	2.30E+00	-2.02E+00	4.29E-02	NA	*Millerozyma*
3.54E+00	-2.82E+00	1.41E+00	-2.00E+00	4.52E-02	NA	*Grosmannia*
3.47E+00	-2.99E+00	1.50E+00	-1.99E+00	4.68E-02	NA	*Cyberlindnera*

Supplementary Table 3: Primers used in this study

Name	Sequence (5' --> 3')	Application
VdAve1_qPCR_Fw	TGTTACCAAAGCAGCACACAAGG	Real-time PCR
VdAve1_qPCR_Rv	CCTTATGCCTCGTTCCCTTCCAC	Real-time PCR
VdGAPDH_Fw	CGAGTCCACTGGTGTCTTCA	Real-time PCR
VdGAPDH_Rv	CCCTCAACGATGGTGAACTT	Real-time PCR
VdAMP3_qPCR_Fw	ATGAAGCTCATTTCTGC	Real-time PCR
VdAMP3_qPCR_Rv	CTAGTTGCAAATGCACAC	Real-time PCR
Chr6g02430_qPCR_Fw	CAGAGCACCACTCACCACAT	Real-time PCR
Chr6g02430_qPCR_Rv	ATCAGGAGTGGCGTGAAGTC	Real-time PCR
ITS1-Fw	AAAGTTTTAATGGTTCGCTAAGA	Real-time PCR
St-Ve1-Rv	CTTGGTCATTTAGAGGAAGTAA	Real-time PCR
NbRUB_Fw	TCCGGGTATTAGCAAAAGCGT	Real-time PCR
NbRUB_Rv	CCCAAGATCTCGGTCAGAGC	Real-time PCR
AtRUB_Fw	GCAAGTGTTGGGTTCAAAGCTGGTG	Real-time PCR
AtRUB_Rv	CCAGGTTGAGGAGTTACTCGGAATGCTG	Real-time PCR
JR2_VdAMP3_LB_Fw	GGTCTTAAUTTTGAGGGGTTCAGCCGATG	To generate VdAMP3 deletion mutant
JR2_VdAMP3_LB_Rv	GGCATTAAUGACGATATGAGTGCTTGCGG	To generate VdAMP3 deletion mutant
JR2_VdAMP3_RB_Fw	GGACTTAAUAATGCTTGAGATGACGACGC	To generate VdAMP3 deletion mutant
JR2_VdAMP3_RB_Rv	GGGTTTAAUCTGCTCACCAAGCCTCCTTC	To generate VdAMP3 deletion mutant
VdAMP3_Comp_Fw	GGGGACAGCTTTCTTGTACAAAGTGGTTTGAGGGGTTCAGCCGATG	To generate VdAMP3 complementation mutant
VdAMP3_Comp_Rv	GGGGACAACTTTGTATAATAAAGTTGCTGCTCACCAAGCCTCCTTC	To generate VdAMP3 complementation mutant
Promoter_VdAMP3_Fw	CTCGGAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCACA CAACATCTATGCTTCAGAAGGTGGCAAAAGTG	To generate pVdAMP3::eGFP transformant
Promoter_VdAMP3_Rv	ATGATGGCCATGTTATCCTCCTCGCCCTTGCTCACCATATTAATTAA GATTGATGGTGTCAAGAGGGTCTGGGATATGATTG	To generate pVdAMP3::eGFP transformant