Faculty of Geotechnical Sciences and Environmental Management

Doctoral Dissertation

Manipulation of the *ACC (1-aminocyclopropane-1-carboxylic acid) deaminase* gene in *Verticillium dahliae* reveals a binary role for ACC in regulating virulence and plant defense: Two sides of the same coin

Maria Dimitra Tsolakidou

Limassol, January 2019

CYPRUS UNIVERSITY OF TECHNOLOGY

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Approval Form

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The approval of the dissertation by the Department of Department of Agricultural Sciences, Biotechnology and Food Science does not imply necessarily the approval by the Department of the views of the writer.

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“The completion of a PhD is the end of an era…but it is also the beginning of a new one!”

Abstract

The maintenance of high agricultural productivity as response to an increasing global demand for food and the exploitation of natural resources have become major challenges in both developed and developing countries. In addition, it is a persistent issue worldwide that a large number of plant pathogens may cause important plant diseases that are responsible for major crop losses. Subsequently, the need to understand the molecular mechanisms underlying fungal pathogenicity is of crucial importance. It has been suggested that some microorganisms, including plant growth promoting rhizobacteria (PGPR), manipulate the level of ethylene in plants by cleaving 1-aminocyclopropane-1-carboxylic acid (ACC), an ethylene precursor, into α-ketobutyrate and ammonia using ACC deaminase. In this thesis, it was investigated whether ACC deaminase of *Verticillium dahliae*, a soil-borne fungal pathogen of many important crops, is involved in pathogenicity of this pathogen. Overexpression of the *V. dahliae* gene encoding this enzyme, labeled as *ACCd*, significantly increased virulence in both tomato and eggplant, while deletion of *ACCd* reduced virulence. Both types of mutant produced more ethylene than the wild type (70V-WT) strain although they significantly differed in ACC content, with overexpressing strains exhibiting lower levels and deletion strains showing higher levels of ACC as compared to the wild type strain. Overexpressing strains were proven to significantly lower the ACC levels in the roots of infected plants while the amount of ACC in the roots of plants infected with deletion mutants increased compared to the wilt type strain. ACC holds a key position in many plant physiological processes with its main role as direct precursor of ethylene. Recent studies have shown that ACC may act as a potential signaling molecule independent from ethylene. To test the hypothesis that ACC acts as a signal for controlling defense, roots of WT and *Never Ripe* (*Nr*) tomato plants and Col-0 and *etr1-1 Arabidopsis* plants were treated with ACC prior to *V. dahliae* inoculation. Plants pre-treated with ACC displayed less severe symptoms than untreated controls. ACC application on the roots of Col-0 and *etr1-1* plants *in vitro* was also found to trigger root hair formation and induce hormone-dependent defense responses. Collectively, our results suggest a novel role of ACC as a regulator of both plant defense and pathogen virulence.

Περίληψη

Η διατήρηση της υψηλής γεωργικής παραγωγικότητας ως απάντηση στην αυξανόμενη παγκόσμια ζήτηση για τροφή και την εκμετάλλευση των φυσικών πόρων, αποτελούν σημαντικές προκλήσεις τόσο στις ανεπτυγμένες όσο και στις αναπτυσσόμενες χώρες. Επιπλέον, είναι ζήτημα παγκόσμιας σημασίας ότι μεγάλος αριθμός παθογόνων μπορεί να προκαλέσει σοβαρές ασθένειες των φυτών, που ευθύνονται για σημαντικές απώλειες των καλλιεργειών και των παραγόμενων προϊόντων. Συνεπώς η κατανόηση των μοριακών μηχανισμών που διέπουν την παθογένεια των φυτοπαθογόνων μικροοργανισμών είναι πολύ σημαντική, ώστε να αναπτυχθούν νέες στρατηγικές αντιμετώπισης τους. Ορισμένοι μικροοργανισμοί έχουν την ικανότητα να μειώνουν τα επίπεδα αιθυλενίου των φυτών, διασπώντας το ACC σε αμμωνία και κετοβουτυρικό οξύ μέσω του ενζύμου ACC απαμινάση. Το ένζυμο ACC απαμινάση αποτελεί έναν από τους κυριότερους μηχανισμούς που διαθέτουν αρκετά ριζοβακτήρια που προάγουν την ανάπτυξη των φυτών καθώς με τη μείωση του παραγόμενου αιθυλενίου τα φυτά σχηματίζουν πλουσιότερο ριζικό σύστημα και μπορούν να ανταπεξέλθουν σε έναν μεγάλο αριθμό αβιοτικών και βιοτικών καταπονήσεων. Η ACC απαμινάση έχει μελετηθεί ευρέως στους ωφέλιμους μικροοργανισμούς, ωστόσο ο ρόλος της στα φυτοπαθογόνα παραμένει άγνωστος. Στην παρούσα διδακτορική διατριβή μελετήθηκε ο ρόλος του γονιδίου *ACC απαμινάση* (*ACCd*) στην παθογένεια και παραγωγή αιθυλενίου του μύκητα *Verticillium dahliae,* ενός σημαντικού εδαφογενούς παθογόνου που προκαλεί μεγάλες απώλειες παραγωγής σε πολλές καλλιέργειες παγκοσμίως. Η υπερέκφραση του γονιδίου *ACCd* στο μύκητα *V. dahliae* οδήγησε σε αύξηση της σοβαρότητας της ασθένειας σε φυτά τομάτας και μελιτζάνας ενώ η απενεργοποίηση του γονιδίου οδήγησε σε σημαντική μείωση της ασθένειας. Και οι δύο τύποι μετασχηματισμένων στελεχών παρήγαγαν περισσότερο αιθυλένιο από το άγριο στέλεχος (70V-WT) παρόλο που διέφεραν σημαντικά ως προς τα επίπεδα ACC, με τα στελέχη υπερέκφρασης να παρουσιάζουν χαμηλότερα επίπεδα ACC και τα απενεργοποιημένα στελέχη υψηλότερα επίπεδα ACC σε σύγκριση με το άγριο στέλεχος. Επιπλέον τα στελέχη υπερέκφρασης του γονιδίου *ACCd* μείωσαν σημαντικά τα επίπεδα ACC στις ρίζες των μολυσμένων φυτών ενώ η ποσότητα του ACC στις ρίζες των φυτών που μολύνθηκαν με τα στελέχη στα οποία είχε διαγραφεί το γονίδιο, ήταν περισσότερη σε σχέση με τα φυτά που είχαν μολυνθεί με το άγριο στέλεχος. Το ACC

είναι ένα μόριο «κλειδί» στη φυσιολογία των φυτών αποτελώντας την πρόδρομη ουσία βιοσύνθεσης του αιθυλενίου. Πρόσφατες μελέτες έχουν δείξει ότι το ACC μπορεί να δρα ως πιθανό μόριο-σηματοδότης ανεξάρτητα από το αιθυλένιο, ρυθμίζοντας την ανάπτυξη των φυτών. Για να διερευνηθεί η υπόθεση ότι το ACC δρα ως μόριο σηματοδότης ενεργοποιώντας τους αμυντικούς μηχανισμούς των φυτών, ρίζες φυτών τομάτας αγρίου τύπου (WT) και *Never Ripe* (*Nr*) και φυτών *Arabidopsis* Col-0 και *etr1-1* μεταχειρίστηκαν με ACC πριν από μόλυνση με το μύκητα *V. dahliae* και η προ μεταχείριση τους με ACC, οδήγησε σε σημαντική μείωση των συμπτωμάτων της ασθένειας. Η εφαρμογή ACC στις ρίζες των φυτών Col-0 και *etr1-1*, *in vitro*, βρέθηκε επίσης ότι προκαλεί αύξηση του μήκους των ριζικών τριχιδίων και επαγωγή μηχανισμών άμυνας μέσω υπερέκφρασης γονιδίων δεικτών. Συλλογικά, τα αποτελέσματα αυτής της διατριβής, υποδεικνύουν ένα δυνητικό ρόλο του ACC, ανεξάρτητο του αιθυλενίου, ως ρυθμιστή τόσο της παθογένειας του μύκητα *V. dahliae* όσο και της επαγωγής μηχανισμών άμυνας των φυτών.

Contents

Chapter 1	General Introduction	1
Chapter 2	Disruption and overexpression of the gene encoding ACC deaminase in the soil-borne fungal pathogen *Verticillium dahliae* reveals a role of ACC as potential regulator of virulence	17
Chapter 3	Investigation of the role of ACC as potential regulator of plant defense against the soil-borne pathogen *Verticillium dahliae*	54
Chapter 4	Summarizing Discussion	81
	References	90
	Thesis deliverables	107

Chapter 1

General Introduction

Due to the continuous population growth, the necessity for maximizing crop productivity is becoming increasingly demanding. The use of synthetic fertilizers and chemicals to combat plant diseases and increase yields, has a negative impact on terrestrial ecosystems and human health (DeLorenzo et al. 2001; Lo 2010; Hartmann et al. 2015; Kim et al. 2017) and often fails to cope with disease outbreaks due to resistance emergence. Plant disease outbreaks have become more often and severe, showing profound social, environmental and economic impacts.

The soil-borne fungus *Verticilliun dahliae* and its infection strategies

Verticillium wilt, caused by the soil-borne fungus *Verticillium dahliae*, is one of the most devastating fungal diseases because of its wide distribution and its ability to survive many years as microsclerotia in the soil (Wilhelm 1955; Fradin and Thomma 2006; Klosterman et al. 2009; Carroll et al. 2018; Shaban et al. 2018). Approximately 400 plant species, including major agricultural crops such as cotton, potato, lettuce, and tomato, annual herbaceous, perennial and woody plants, are susceptible to *V. dahliae* resulting in extensive economic losses (Pegg and Brady 2002; Fradin and Thomma 2006; Klosterman et al. 2009; Luo et al. 2014; Keykhasaber et al. 2017; Carroll et al. 2018). Verticillium wilt disease management is very difficult due to its broad host range and microsclerotia formation and has been relying on soil fumigation which has tremendous economic and environmental costs inconsistent with sustainable agriculture (Klosterman et al. 2009; Carroll et al. 2018). After the ban of methyl bromide, the only available control methods are preventive measures such as the use of tolerant varieties, biological control agents, crop rotation and soil solarization. However, once plants are infected by the fungus no measures exist to prevent progression of the disease (Fradin and Thomma 2006; Yadeta and J. Thomma 2013; Carroll et al. 2018).

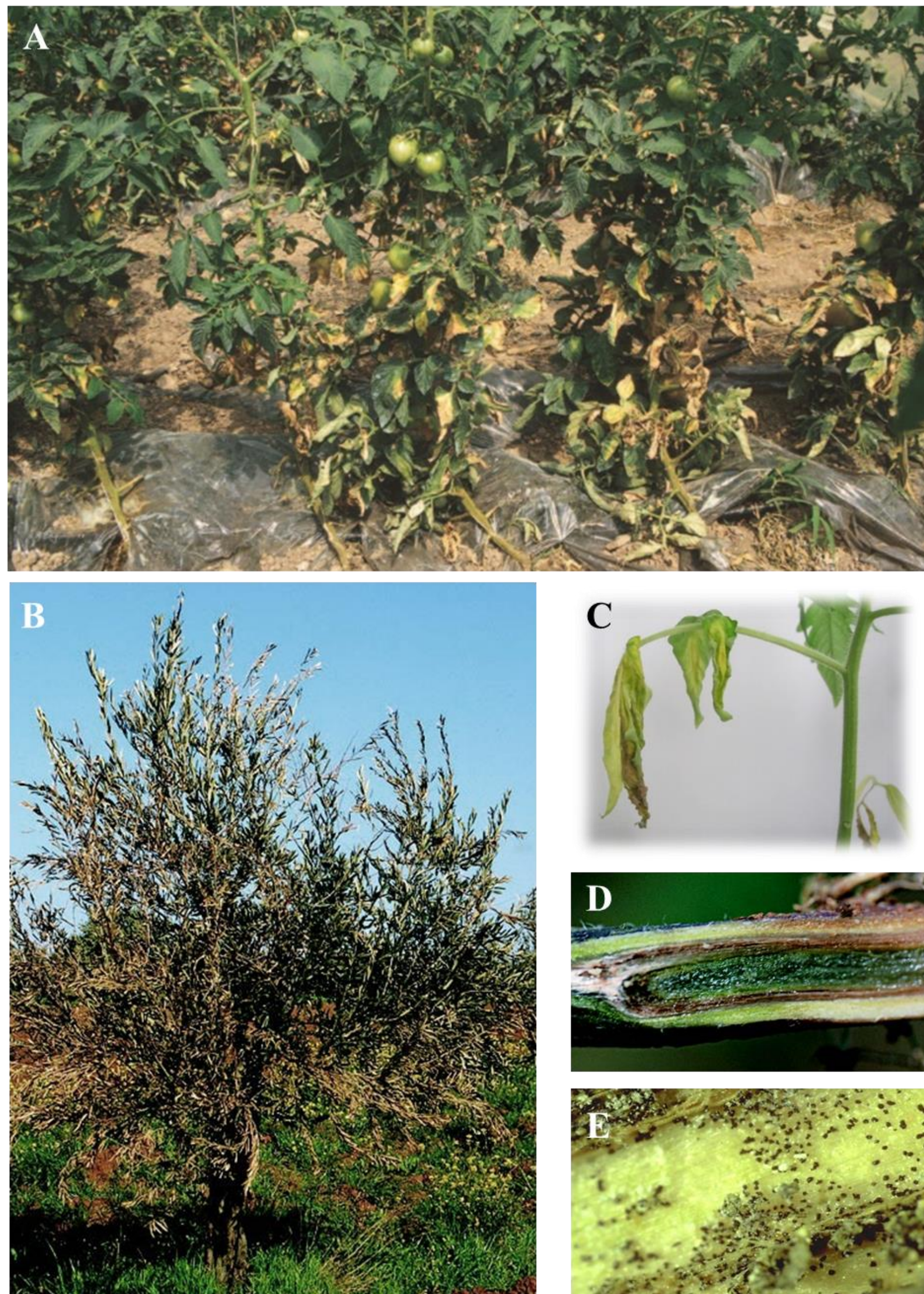


Fig. 1. Symptoms and morphological characteristics of *Verticillium dahliae.* **(A)** Symptoms of leaf necrosis and defoliation in tomato. **(B)** Partial dieback of shoots and branches in olive tree. **(C)** Chlorosis and wilting of tomato leaves. **(D)** Longitudinal section of the stem base of a tomato plant infected by *V. dahliae* displaying severe brown vessel discoloration. **(E)** Formation of microsclerotia by *V. dahliae* (Photographs A, B and D were reproduced from Tjamos E. C., 2005, Phytopathology, Stamoulis Press, Athens and photograph E from Berlanger and Powelson, 2000. Verticillium wilt. *The Plant Health Instructor*. DOI: 10.1094/PHI-I-2000-0801-01).

V. dahliae attacks its hosts through the root system and penetrates the endodermis at the root tip and/or at the sites of lateral root formation. During colonization of the xylem the fungus proliferates, inhibiting water transport which results in wilting (Bishop and Cooper 1983; Fradin and Thomma 2006; Yadeta and J. Thomma 2013; Carroll et al. 2018). To overcome the physical barriers of the plants and facilitate infection, *V. dahliae* has evolved complex virulence mechanisms which are controlled by multiple signaling pathways (Klosterman et al. 2011; Luo et al. 2014; Shaban et al. 2018). The pathogen utilizes a variety of substances such as cell wall-degrading enzymes (CWDE), toxins, elicitors and other metabolites (Luo et al. 2014). To colonize its host, the first step is the adhesion to the root system and subsequently the penetration of the host plant. In order to attach to plant roots, *V. dahliae* regulates the expression of genes that encode putative adhesion-like proteins, that control root adhesion and colonization processes. Deletion of the *Vta2* gene in *V. dahliae*, a transcription activator of adhesion, produced mutants that were unable to colonize plants and induce disease symptoms (Tran et al. 2014). *V. dahliae* mutant strains compromised in the *VdPls1* gene, encoding a tetraspanin, and the *VdNoxB* gene, encoding a catalytic subunit of membrane-bound NADPH oxidases for reactive oxygen species (ROS) production, were unable to form penetration pegs and therefore failed to initiate colonization in cotton plants (Zhao et al. 2016).

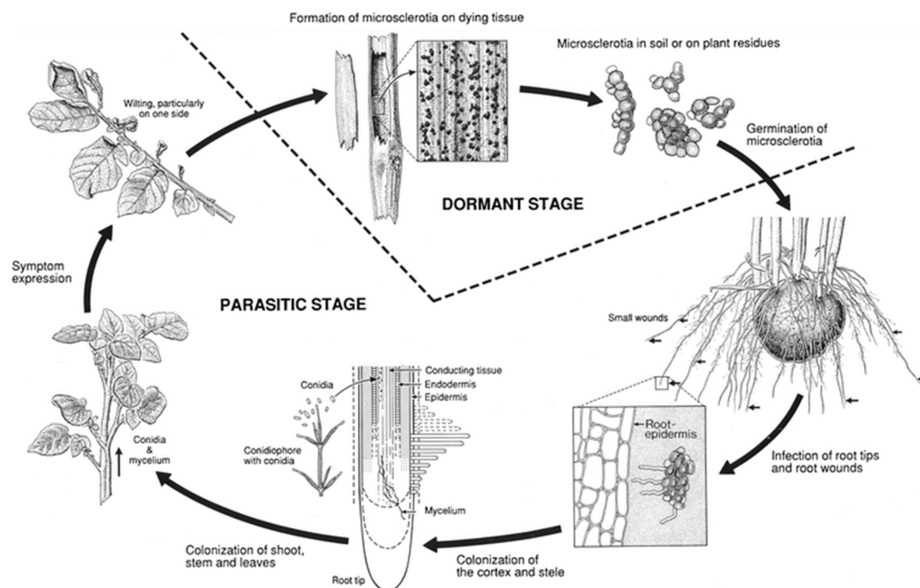


Fig. 2. Disease cycle of *Verticillium dahliae* in potato (reproduced from Keykhasaber et al., 2017).

The next natural barrier the pathogen has to overcome for successful colonization, is the plant cell wall. Penetration of the host plants by *V. dahliae* is facilitated through the production of CWDE which are used to depolymerize plant cell wall components. Several studies have demonstrated the importance of CWDE, such as endoglucanases, polygalacturonases, pectinases, and cellulases, in the expression of disease symptoms and pathogenesis (Puhalla and Howell 1975; Carder et al. 1987; Durrands and Cooper 1988a, 1988b; Maruthachalam et al. 2011; Liu et al. 2013; Zhang et al. 2015). Additionally, genes related to CWDE, like the *Sucrose Non Fermenting 1 protein kinase* gene (*SNIF*), indirectly affect the regulation of CWDE and play an important role in virulence (Tzima et al. 2011).

Once *V. dahliae* enters the host, it gets access into xylem vessels and starts to propagate quickly in order to translocate to neighboring vascular and cortical tissues (Fradin and Thomma 2006; Luo et al. 2014; Shaban et al. 2018). In this phase, production of proteins that participate in signal transduction, such as G proteins, MAP kinases and protein kinases A and secretion of toxins play a critical role in *V. dahliae* disease development. Deletion of genes involved in these signal transduction pathways resulted in reduced virulence, conidiation and ethylene production by the fungus (Tzima et al. 2010a, 2012). The phytohormone ethylene is another factor that has been suggested to play a role in Verticillium wilts, as it is considered to be one of the main contributing factors to symptoms development such as epinasty, stunting, premature senescence and leaf abscission (Wiese and Devay 1970; Cronshaw and Pegg 1976).

Plant defense mechanisms against *Verticillium dahliae*

Plant defense mechanisms against *V. dahliae* consist of an array of structural barriers, production of antimicrobial molecules and the induction of multifaceted molecular changes (Fig. 3).

In order to facilitate infection, *V. dahliae* has to overcome several mechanical and chemical plant defense responses. Suberin deposition in root endodermis, during normal root development, the quick deposition of lignin in the prevascular stages of infection and the formation of lignin tubers (papilla) around penetrating hyphae act as natural barriers against *V. dahliae* (Talboys 1958; Griffith 1971). However, if the pathogen overcomes these prevascular defense responses, suberin deposition on vascular cells and the formation of outgrowths on parenchyma cells of xylem vessels, called tyloses,

prevent the spread and movement of the fungus (Robb et al. 1989; Benhamou 1995). At the same time, the production of antimicrobial compounds such as Pathogenesis-Related proteins (PR), phytoalexins and phenolic compounds are used by the plants to eliminate the pathogen (Talboys 1958; Benhamou 1995; Gold and Robb 1995; Daayf et al. 1997; Williams et al. 2002). Elemental Sulphur, terpenoid phytoalexins and gossypols are among the phytoalexins that have been found in cotton and tomato cultivars and have been implicated in *Verticillium* resistance (Cooper et al. 1996; Williams et al. 2002; Gao et al. 2013).

To protect themselves against pathogens, plants have also developed a network of signaling molecules involving hormones and resistance related genes. Plant hormones play a vital role in the induction of plant defense mechanisms against various pathogens such as fungi, pests, bacteria and viruses. Among plant hormones Salicylic Acid (SA), Jasmonic Acid (JA) and Ethylene (ET) are the main players against *V. dahliae* (Shaban et al. 2018). The SA-mediated signaling pathway is mainly triggered against biotrophic pathogens, however it has been implicated in defense against hemi-biotrophic fungi, like *V. dahliae* (Glazebrook 2005). Strong evidence for the role of SA in defense against *V. dahliae* was provided recently by Liu et al. (2014). In this study two isochorismatase effectors, VdISC1 and PsISC1, secreted by *V. dahliae* and *Phytophthora sojae* respectively, were found to suppress SA accumulation in their host. In addition, deletion of the genes encoding these effectors resulted in reduced disease symptoms, increased SA level and induced expression of PR1 in cotton. JA signaling pathway has also been studied excessively in several plant species and has been found to play an important role in early defense responses against *V. dahliae* (Thaler and Higging 2004; Tjamos et al. 2005; Fradin et al. 2011; Gao et al. 2013). JA deficient tomato and JA-insensitive Arabidopsis plants were found to suffer more severely from *Verticillium* infections (Thaler and Higging 2004; Tjamos et al. 2005) while a substantial number of candidate genes involved in different pathways have been proposed to induce defense responses of cotton against *V. dahliae* and increase the JA level following pathogen infection (Zhang et al. 2013; Xu et al. 2014). The role of ethylene in disease resistance against *V. dahliae* is rather complex as apart from its ambiguous contribution to resistance is also considered to participate in symptom expression (Cronshaw and Pegg 1976; Pegg 1976; Pegg and Cronshaw 1976; Thomma et al. 1999; Pantelides et al. 2010b, 2010a). Pantelides et al. (2010a) studied the ethylene signal pathway in Arabidopsis after

infection with *V. dahliae* and found that only the ethylene insensitive mutant *etr1-1* is associated with resistance to *V. dahliae*, accompanied with enhanced expression of PR genes such as *PR-2*, *PR-5*, *CHI-1*, *CHI-2* and the transcription factor *Myb75*. Similarly, another ethylene mutant, *ein3-1*, also enhanced the resistance against Verticillium wilt (Fradin et al. 2011), but conversely similar regulatory factors such as *ein2-1*, *ein4-1* and *ein6-1* were related to susceptible response in Arabidopsis (Johansson et al. 2006).

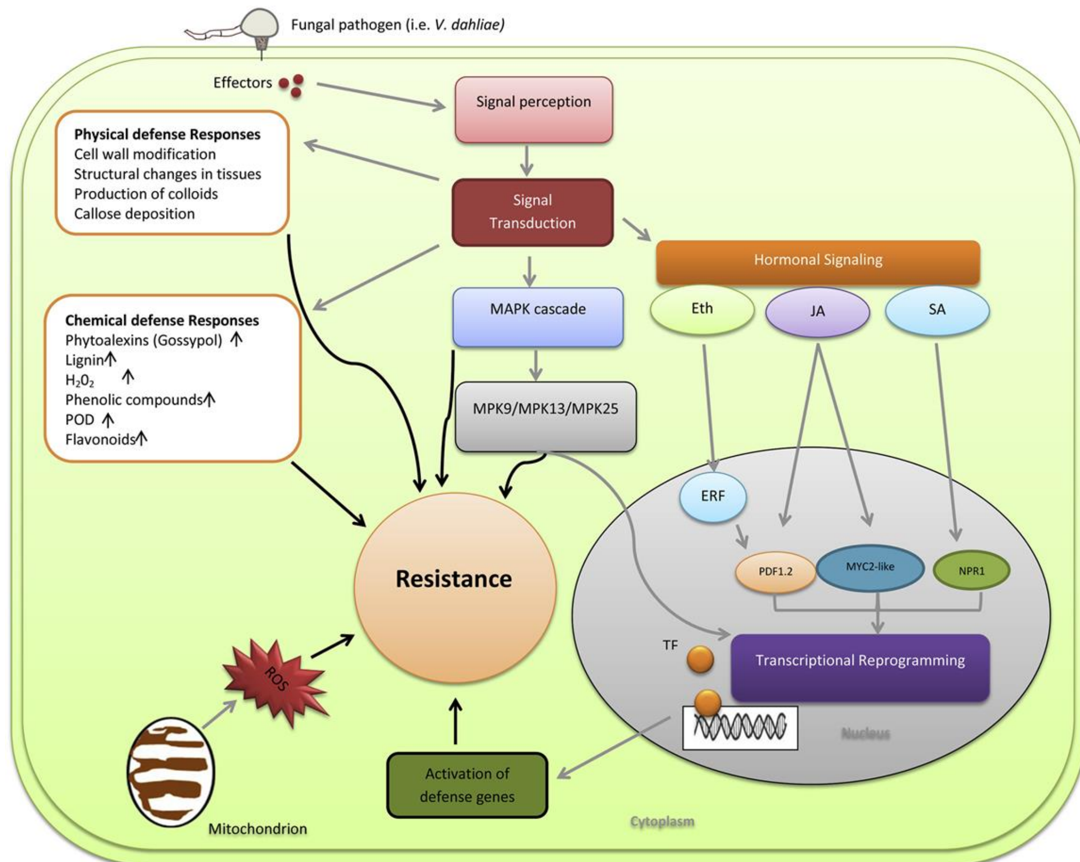


Fig. 3. Molecular mechanisms of resistance to Verticillium wilt in cotton. As the cotton plant is infected by *V. dahliae*, a cascade of reactions involving physical, chemical and molecular responses become activated, which in turn fine tune the resistance response. **Eth**: Ethylene, **JA**: Jasmonic acid, **SA**: Salicylic acid, **ERF**: Ethylene Responsive Factor, **PDF1.2**: Plant Defensin 1.2, **MYC2**: MYC-related transcriptional activator, **NPR1**: Natriuretic Peptide Receptor 1, **TF**: Transcription factors, **MAPK**: Mitogen-activated protein kinase, **POD**: Peroxidase (reproduced from Shaban et al., 2018).

Resistance against *Verticillium* spp. has been identified in several plant species, including cotton, potato and strawberry. However, a locus responsible for resistance against Verticillium has been cloned only from tomato. The Ve locus, which provides resistance to race 1 strains of *V. dahliae* and *V. albo-atrum*, transcribes two closely

linked inversely oriented genes *Ve1* and *Ve2* that encode leucine-rich repeat of receptor-like proteins for Ve1 and Ve2 proteins, respectively (Kawchuk et al. 2001; Fradin et al. 2009). Both *Ve1* and *Ve2* confer resistance to Verticillium wilt in potato, but in tomato and Arabidopsis only *Ve1* is responsible for resistance to *V. dahliae* (Kawchuk et al. 2001; Fradin et al. 2011).

Ethylene in plant-microbe interactions

Ethylene is a gaseous plant hormone that regulates a number of physiological and developmental processes in plants, such as seed germination, leaf and flower senescence and abscission, fruit ripening, root elongation and branching (Abeles et al. 1992). Ethylene also functions as a stress hormone, as its production is elicited in response to several abiotic and biotic stresses, such as wounding, flood, salinity and pathogen attack (Abeles et al. 1992; Johnson and Ecker 1998). In plants, ethylene is synthesized from methionine (Fig. 3A) which is first converted to S-adenosyl methionine (S-AdoMet or SAM) by SAM synthase, then SAM is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS), and finally ethylene is produced from ACC by ACC oxidase (ACO) (Adams and Yang 1979). Except for ethylene production, ethylene perception and signaling in plants is important for the induction of plant defense mechanisms in response to external stimuli. The molecular genetic dissection of ethylene signaling has been described in the model plant *Arabidopsis thaliana* providing important information in understanding how ethylene interferes not only in plant physiological processes but also in induction of defense mechanism against abiotic and biotic challenges. In this model, which is highly conserved in plants (Ju and Chang 2015), the ethylene molecule represses ethylene receptor signaling and thereby the negative regulator CTR1 (constitutive triple response 1). CTR1 repression relieves the inhibition of EIN2 (ethylene insensitive 2), a positive regulator of ethylene signaling which initiates a transcriptional cascade involving ethylene responsive transcription factors (ERFs), resulting in ethylene-mediated stress responses in plants (Johnson and Ecker 1998).

The role of ethylene in biotic stresses is quite controversial and can influence plant-pathogen interactions in different ways, as it can either trigger plant defense responses or aggravate disease symptoms (Pegg and Cronshaw 1976; Pegg 1976; Lund et al. 1998; Thomma et al. 1999; Van Loon et al. 2006; Pantelides et al. 2010b, 2010a). The

effect ethylene may have on disease resistance or symptoms expression depends on a wide range of factors, including the timing of ethylene exposure, the physiological state of the host as well as the lifestyle of the pathogen. For example, ethylene production by plants at early stages of infection has been associated with the induction of defense mechanisms, while its production during infection may act in favor of the pathogen, contributing to symptom development (Van Loon et al. 2006). Exogenous application of ethylene on carrots resulted in enhanced resistance against *Botrytis cinerea* (Hoffman et al. 1988). In Verticillium wilt–tomato disease interactions, fumigation of intact tomato plants with ethylene immediately following inoculation with *V. albo-atrum* and before the establishment of the pathogen resulted in impaired symptom development (Pegg 1976). However ethylene also acts as a toxin synergist since its exogenous application on tomato plants enhanced wilting symptoms caused by *V. albo-atrum* toxins (Cronshaw and Pegg 1976). Moreover the availability of transgenic ethylene-insensitive plants resulting from mutations in the ethylene-perception pathway has indicated that ethylene can influence particular plant-pathogen interactions in different ways. The *ein2* (ethylene-insensitive) mutation in *Arabidopsis* has been shown to decrease the symptoms of two bacterial pathogens, *Pseudomonas syringae* pv. tomato and *Xanthomonas campestris* pv. campestris (Bent et al. 1992), but increased susceptibility to *Botrytis cinerea* (Thomma et al. 1999). Analysis of *Arabidopsis* ethylene insensitive mutants against *V. dahliae* revealed enhanced resistance in *etr1-1* (ethylene receptor mutant) plants, but not in other ethylene-deficient mutants, indicating a crucial role of ETR1 in defense against this pathogen and pointing out the different effects ethylene perception may have in Verticillium wilt disease development (Pantelides et al. 2010a).

Besides plants, several phytopathogenic fungi and bacteria are able to produce ethylene, reinforcing their ability to colonize plant tissues. In microorganisms, ethylene biosynthesis may occur via three different pathways (Fig. 4). It may either start from methionine involving ACC as an intermediate and/or α-keto-γ-methylthiobutyric acid (KMBA) or from 2-oxoglutarate (OXO) which is catalyzed by a multifunction ethylene forming enzyme (EFE) into ethylene (Primrose 1977; Fukuda et al. 1986; Hottiger and Boller 1991; Jia et al. 1999; Chagué et al. 2002).

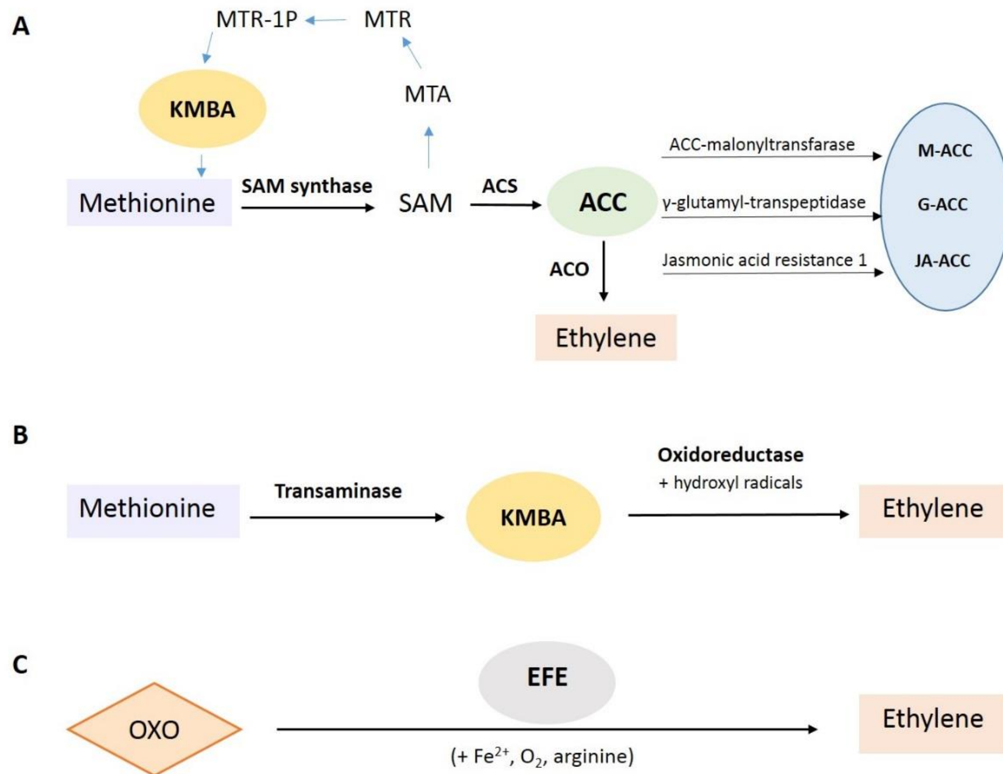


Fig. 4. Ethylene biosynthesis pathways. (A) The ACC-mediated biosynthetic pathway and ACC conjugation process. Methionine is converted to SAM by the enzyme SAM synthase. SAM is converted to ACC and MTA, by the enzyme ACS. MTA is reconverted to methionine by a series of biochemical reactions, described as the Yang cycle. The enzyme ACO catalyzes the conversion of ACC to ethylene. In addition, ACC can be conjugated to M-ACC, G-ACC or JA-ACC by the action of the enzymes ACC-malonyl transferase, g-glutamyl-transpeptidase and jasmonic acid resistance 1, respectively**. (B)** The KMBA-mediated biosynthetic pathway. Methionine is deaminated to KMBA by a transaminase, and KMBA is further oxidized to ethylene. The oxidization is facilitated in a nonenzymatical way and requires hydroxyl radicals that are produced from molecular oxygen by a Fe(III)EDTA-oxidoreductase. **(C)** The OXO-mediated biosynthetic pathway. OXO is catalyzed into ethylene by EFE in the presence of other amino-acids as cofactors. **SAM**: S-adenosylmethionine, **ACC**: aminocyclopropane-1-carboxylic acid, **ACS**: aminocyclopropane-1-carboxylic acid synthase, **ACO**: aminocyclopropane-1-carboxylic acid oxidase, **MTA**: 5 methylthioadenosyl, **MTR**: 5-methylthioribose, **MTR-1P**: 5-methylthioribose-1-phosphate, **KMBA**: a-keto-γ-methylthiobutyric acid, **OXO**: 2-oxoglutate, **EFE**: Ethylene Forming Enzyme, **M-ACC**: malonyl-ACC, **G-ACC**: glutamyl-ACC, **JA-ACC**: jasmonic acid-ACC.

Potential roles of ethylene production and perception by pathogens, in virulence and disease development have been studied in a few fungal plant pathogens including *V. dahliae* (Tzeng and Devay 1985; Cristescu et al. 2002; Al-Masri et al. 2006; Tzima et al. 2010, 2012, Zhu et al. 2012, 2017). Ethylene perception by *Colletotrichum*

gloeosporioides is required for spore germination and appressorium formation (Kim et al. 2000). Ethylene production by *Alternaria alternata* that occurs via the KMBA pathway, promotes spore germination and hyphal growth of the fungus and is associated with enhanced virulence (Kępczyńska 1994; Zhu et al. 2017). In *Botrytis cinerea* ethylene activates transcriptional changes of fungal genes, some of which may play a role in fungus-plant interaction (Chagué et al. 2006) and it is reported to modulate both hyphal growth and pathogenesis in *B. cinerea* - tomato and *B. cinerea* - grape pathosystems (Cristescu et al. 2002; Zhu et al. 2012). However, it has been reported that ethylene may have different effects on growth and development of *B. cinerea*, such as conidial germination, germ tube elongation and mycelial growth, at different developmental stages and in different systems (Zhu et al. 2012). Ethylene has also been involved in the development of *Verticillium* wilt symptoms, such as epinasty, stunting, premature senescence and leaf abscission, as the symptoms caused by this pathogen are similar to the symptoms caused to the plants upon ethylene exposure (Wiese and Devay 1970; Cronshaw and Pegg 1976). For example symptoms on potato leaves after treatment with *Verticillium* toxins were similar to those observed upon treatment of plants with ethylene (Mansoori and Smith 2005). However, it remains unclear whether ethylene produced by *V. dahliae* is a virulence factor.

ACC: More than just the precursor of ethylene

ACC is best known as the direct precursor of ethylene in plants and is considered to be the rate-limiting step in ethylene biosynthesis (Adams and Yang 1979; Yang and Hoffman 1984). Except for its role in regulating ethylene biosynthesis through ACS and ACO activity, ACC can also form conjugates which are considered to control the available ACC pool in plants (Fig. 4A). ACC forms three conjugates, malonyl-ACC (MACC), γ-glutamyl-ACC (GACC) and jasmonyl-ACC (JA-ACC), but their biological role is not fully known (Amrhein et al. 1981; Hoffman et al. 1982; Martin et al. 1995; Staswick and Tiryaki 2004). MACC is considered to control the ACC pool as it can be translocated between the cytocol and vacuoles and it can be also reconverted to ACC (Tophof et al. 1989). The role of GACC has not yet been elucidated while JA-ACC, a conjugate of jasmonic acid with ACC, is suggested to function as a modulator of the hormonal crosstalk between ethylene and jasmonic acid (Staswick and Tiryaki 2004; Vanderstraeten and Van Der Straeten 2017). Unlike ethylene which due to its gaseous

nature can cause mainly local responses, ACC and its conjugate MACC can cover either short distances (intracellular and intra-tissue) or long distances (via the xylem and phloem) within a plant (Bradford and Yang 1980). Furthermore, ACC can be transported not only within the tissues of the plants but can also be exuded by seeds and roots (Penrose and Glick 2001) highlighting the role of ACC as an internal and external signaling molecule (Van de Poel and Van Der Straeten 2014; Nascimento et al. 2018).

Interestingly, recent studies have suggested a different but equally important role for ACC as a signaling molecule, acting independently from ethylene (Yoon and Kieber 2013; Van de Poel and Van Der Straeten 2014; Vanderstraeten and Van Der Straeten 2017; Nascimento et al. 2018). For example ACC can be secreted by plant roots or seeds into the rhizosphere and act as a signal for the recruitment of beneficial bacteria that use ACC as a source of nitrogen (Glick 2014; Nascimento et al. 2018). ACS octuple mutants of *Arabidopsis* generated by Tsuchisaka et al. (2009) displayed embryo lethality and impaired root branching which were not observed in ethylene signaling mutants. Further evidence for the role of ACC as a signaling molecule was obtained by studies showing that root cell elongation in *A. thaliana* appears to be controlled by ACC, in a non-ethylene dependent way (Xu et al. 2008; Tsang et al. 2011). According to these observations, a signaling role for ACC independent from ethylene is highly likely. However whether ACC or one of its derivatives serve as a signal, is still a matter of debate and needs further investigation.

Ethylene and ACC modulation by microorganisms

Plant-associated microorganisms may have an impact on plant stress ethylene, co-regulate ethylene and also perceive ethylene, potentially responding to it. Microorganisms have developed an array of mechanisms to modulate plant ACC and ethylene levels, either by producing compounds, enzymes and effectors that affect the production and signaling of plant ethylene and ACC, or alternatively by contributing to ethylene production by directly synthesizing ethylene for their own benefit (Fig. 5).

The chemical compounds rhizobitoxine (RTX), an enol-ether amino acid, and aminoethoxyvinylglycine (AVG) act as inhibitors of the plant ACS (Icekson and Apelbaum 1983; Yasuta et al. 1999) and are used by microorganisms to reduce ethylene levels produced by plants. The legume root nodulating bacterium *Bradyrhizobium elkanii*, that is an RTX producer, enhances the nodulation process and nodulation

competitiveness by inhibiting ethylene biosynthesis in host roots (Okazaki et al. 2004), while the plant pathogenic bacterium *Burkholderia andropogonis* also produces RTX presumably to manipulate ethylene levels and facilitate infection (Mitchell and Frey 1988). Plant pathogens also produce effectors targeting ethylene biosynthesis and signaling pathways. For example the plant pathogen *Xanthomonas euvesicatoria* produces a type III secretion effector (Xopd) which directly targets the tomato ethylene responsive transcription factor SlERF4 to suppress ethylene production, which is required for anti-Xcv immunity and induction of ethylene defense responses (Kim et al. 2013).

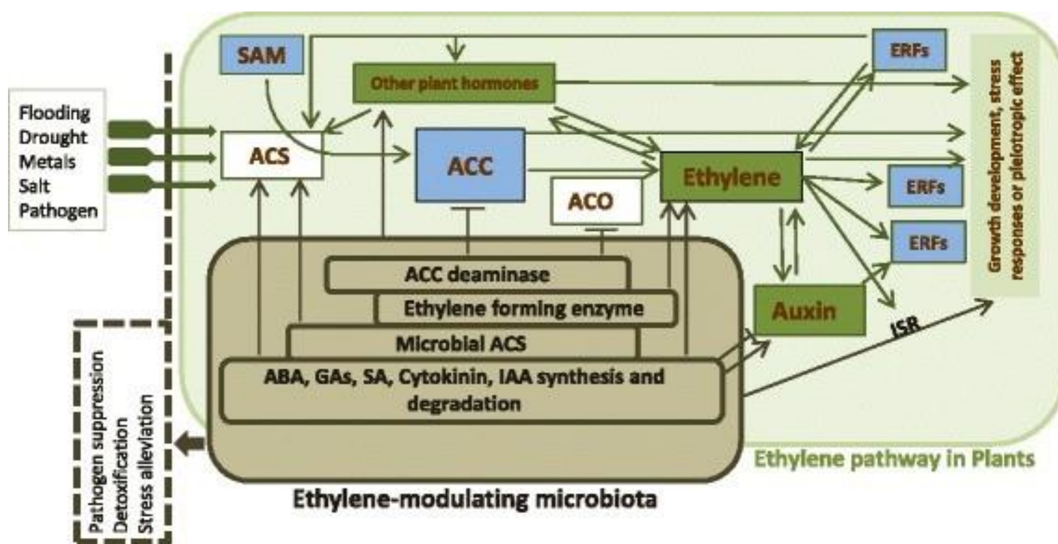


Fig 5. A holobiont-level regulation of ethylene signaling and plant stress response. Ethylene pathway in plants (green area). ACC is synthesized from SAM by the action of ACS. ACC is then converted to ethylene by the enzyme ACO, triggering different ethylene response factors (ERFs). Plant-associated microorganisms can alter virtually all steps of ethylene signaling. Some species can increase ethylene levels by producing ACO (microbial ethylene-forming enzyme), by inducing ACS in plant or by affecting other plant hormones indirectly. They can also modulate ethylene response by producing plant hormones that interact with ethylene signaling. Other microorganisms can also decrease ethylene production by cleaving its precursor ACC. White boxes show ethylene biosynthetic enzymes, green boxes show plant hormones and signals, and blue boxes show the molecules involved in the ethylene pathway. **ABA**: abscisic acid, **GA**: gibberellic acid, **SA:** salicylic acid, **SAM**: S-adenosylmethionine, **ACC**: aminocyclopropane-1-carboxylic acid, **ACS**: aminocyclopropane-1-carboxylic acid synthase, **ACO**: aminocyclopropane-1-carboxylic acid oxidase, **ERFs**: ethylene responsive factors (reproduced from Ravanbakhsh et al. 2018).

Microorganisms can also decrease plant ethylene levels, by producing the enzyme ACC deaminase which cleaves ACC into α-ketobutyrate and ammonia (Honma and Shimomura 1978). This is a well characterized trait of several plant growth promoting bacteria (PGPB) that utilize ACC deaminase probably to get access to the plant carbon and nitrogen sources they need, but at the same time they lower ACC levels in plants, which in turn prevents ethylene growth inhibition and promotes plant growth (Glick et al. 1998; Penrose et al. 2001). However, the ACC deaminase gene has been found in plant pathogens too, although its role has not yet been elucidated. The model described by Glick et al. (1998) for plant ethylene reduction by ACC-deaminase producing PGPB suggests that in response to the presence of tryptophan and other small molecules in plant root exudates, ACC deaminase-producing PGPB secrete the phytohormone indole-3-acetic acid (IAA), that can be partly taken up by plants. IAA induces the transcription of *ACS* in plants that catalyzes the formation of ACC and along with other small molecules in root exudates, some of the plant ACC is exuded from seeds and roots, taken up by the PGPB, and subsequently cleaved by ACC deaminase (Fig. 6). As a consequence, the plant ACC pool is deprived and thus plant ethylene levels decline.

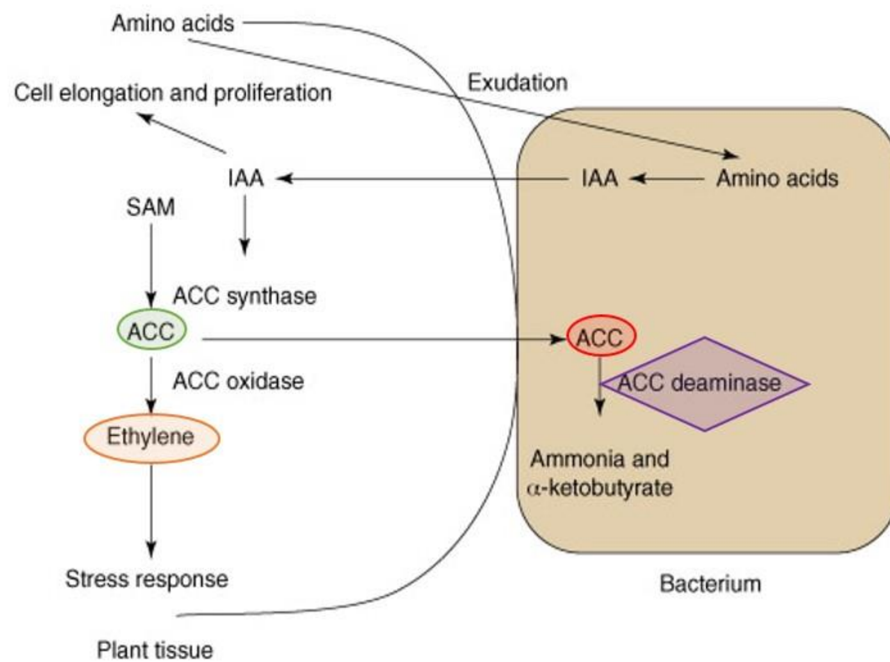


Fig. 6. Schematic representation of how bacteria with ACC deaminase activity lower the ethylene concentration and thereby prevent ethylene-caused inhibition of root elongation. **IAA**: indole acetic acid, **SAM**: S-adenosylmethionine, **ACC**: aminocyclopropane-1-carboxylic acid, (reproduced from Glick et al. 1998) .

Plant microbes can also increase plant ethylene levels by directly producing ethylene, this way contributing to the total ethylene of the plant-microbe system, or by inducing plant ACS activity. Rhizosphere microbes can increase plant ethylene indirectly by secreting auxin and/or cytokinin that up-regulate the expression of *ACS*-coding genes (Patten and Glick 2002; Veselov et al. 2003). Plant pathogens can also manipulate plant ethylene levels to facilitate infection. Ethylene production by *Ralstonia solanacearum* induced premature ripening in banana (Freebairn and Buddenhagen 1964), while ethylene production by *Botrytis cinerea* contributed to ethylene production in the *B. cinerea*–grape system (Zhu et al. 2012).

Thesis outline

The soil-borne fungus *Verticillium dahliae* is among the most destructive plant pathogens that affect many crops and ornamentals in different regions of the world (Pegg and Brady 2002; Yadeta and J. Thomma 2013; Inderbitzin and Subbarao 2014). Management of Verticillium wilt is rather difficult due to the sustained viability of microsclerotia, its broad host range, the unavailability of effective fungicides, and the lack of effective genetic resistance (Tjamos 1989; Klosterman et al. 2009; Carroll et al. 2018). Therefore the detailed study of virulence mechanisms in *V. dahliae* is essential in order to understand how this pathogen manipulates host defense responses to facilitate infection. This information would provide further evidence to develop comprehensive and effective control strategies. One of the factors that has been correlated to the symptoms caused by *V. dahliae* is the plant hormone ethylene, since the symptoms associated with *V. dahliae* such as epinasty, chlorosis and leaf abscission can be reproduced or worsened by plant exposure to ethylene (Wiese and Devay 1970; Cronshaw and Pegg 1976; Pegg 1976; Mansoori and Smith 2005). Although it has been shown that ethylene production by *V. dahliae* occurs via the KMBA pathway (Tzeng and DeVay 1984), genes involved in the ACC-mediated pathway such *ACC synthase* and *ACC deaminase* have been identified in the genome of *V. dahliae*, but their role in pathogenicity and ethylene production has not been investigated.

The main goal of this thesis was to investigate the role of *ACC deaminase* gene of the soil-borne fungus *V. dahliae* in its pathogenicity, ACC and ethylene production.

In **Chapter 2,** the native *ACC deaminase* (*ACCd*) gene of the fungus was manipulated in order to alter ACC levels and consequently ethylene production. The resulting transformed strains were evaluated for their virulence under different plant growth conditions, their morphological characteristics and their ability to affect ACC and ethylene levels. It was shown that both *ACCd* overexpression and deletion mutants produced more ethylene than the wild type strain although they significantly differed in ACC content, with overexpressing strains exhibiting lower levels and deletion strains showing higher levels of ACC as compared to the wild type strain. In addition, *ACCd* overexpression led to enhanced virulence of *V. dahliae* while *ACCd* deletion resulted in impaired disease development, indicating that accumulation or reduction of pathogen-derived ACC in the roots might be involved in the outcome of infection.

Results from Chapter 2 suggested a role of ACC as negative virulence regulator. However, some recent studies suggested a role for ACC as a signaling molecule, thus in **Chapter 3** it was investigated whether ACC could act as positive regulator of plant defense and whether this function is ethylene independent. To test this hypothesis, *ACCd* mutants were initially assessed for their ability to alter the ACC levels in the roots of tomato plants. Subsequently pathogenicity assays were performed in WT and ethylene insensitive tomato and Arabidopsis plants pre-treated with ACC in order to test whether accumulation/application of ACC in the roots of plants had an effect on disease severity. It was also investigated whether ACC could induce defense responses in the roots of Arabidopsis plants. The results obtained suggest that ACC acts as positive defense regulator against Verticillium wilt disease.

Finally, in **Chapter 4** the main findings of this work are summarized and the main conclusions of the thesis are briefly discussed. Moreover, a hypothesis regarding the role of ACC deaminase in pathogenicity of *V. dahliae* and the role of ACC in the outcome of infection is provided along with future perspectives.

Chapter 2

Disruption and overexpression of the gene encoding ACC deaminase in the soil-borne fungal pathogen *Verticillium dahliae* revealed a role of ACC as potential regulator of virulence

A slightly modified version of this chapter has been published to Molecular Plant-Microbe Interactions (DOI: 10.1094/MPMI-07-18-0203-R)

Abstract

It has been suggested that some microorganisms, including plant growth promoting rhizobacteria (PGPR), manipulate the level of ethylene in plants by degrading 1-aminocyclopropane-1-carboxylic acid (ACC), an ethylene precursor, into α-ketobutyrate and ammonia using ACC deaminase. By reducing ethylene, plants form longer roots and become more resistant to a variety of abiotic and biotic stresses. Despite the beneficial effect of ACC deaminase-producing microorganisms, the role of ACC deaminase in plant pathogens has been poorly studied. Here, we investigated whether ACC deaminase of the soil-borne pathogen *Verticillium dahliae* is involved in pathogenesis and ethylene production by the fungus. Contrary to the plant growth promoting function of ACC deaminase conferred by PGPR, overexpression of the *V. dahliae* gene encoding this enzyme, labeled as *ACCd*, significantly increased virulence in both tomato and eggplant, while disruption of *ACCd* reduced virulence. Microsclerotia formation was reduced by both types of mutation, but other growth and developmental traits were not significantly affected. Interestingly, both types of mutant produced more ethylene than wild type (70V-WT) strain although they significantly differed in ACC content. Collectively, our results suggest that the role of *ACCd* in *V. dahliae'*s virulence is related to fungal ACC regulation, rather than ethylene production.

Introduction

Verticillium dahliae is a soil-borne fungal pathogen that causes Verticillium wilt disease in a wide variety of economically important annual herbaceous, perennial, and woody plants (Bhat and Subbarao 1999; Fradin and Thomma 2006; Keykhasaber et al. 2017). Due to the broad host range, the ability to survive many years as microsclerotia in the soil, and the very limited availability of control options, *V. dahliae* continuously causes severe crop losses in many temperate and subtropical regions around the world (Tjamos 1989; Robison et al. 2001b; Fradin and Thomma 2006). *V. dahliae* evolved various mechanisms to overcome plant defense systems and facilitate *in planta* proliferation (Klosterman et al. 2009; Maruthachalam et al. 2011). Secretion of a variety of molecules, including cell wall-degrading enzymes, phytotoxins and effectors, is one such mechanism (Tzima et al. 2011; Luo et al. 2014). Certain regulatory proteins that control key signal transduction pathways, such as GTP-binding proteins, MAP kinases and protein kinases A, also play critical roles in virulence (Tzima et al. 2010, 2012).

Ethylene is a gaseous plant hormone that affects developmental processes such as flowering, senescence, fruit ripening, and leaf abscission and also participates in regulating responses to biotic and abiotic stresses (Abeles et al. 1992; Johnson and Ecker 1998). Certain bacteria and fungi also produce ethylene presumably to manipulate plant ethylene-mediated regulation of these processes in a manner that facilitates their colonization of plants. Potential roles of pathogen-produced ethylene in virulence and disease development have been studied in *V. dahliae* and a few other pathogens (Tzeng and Devay 1985; Cristescu et al. 2002; Al-Masri et al. 2006; Tzima et al. 2010, 2012, Zhu et al. 2012, 2017). Ethylene may be involved in causing Verticillium wilt symptoms, such as epinasty, stunting, premature senescence and leaf abscission (Wiese and Devay 1970; Cronshaw and Pegg 1976). Ethylene perception is required for spore germination and appressorium formation in *Colletotrichum gloeosporioides* (Kim et al. 2000). In *Botrytis cinerea*, ethylene affected transcriptional reprogramming of the genes that may be involved in plant interaction (Chagué et al. 2006). However, its role in plant infection remains unclear (Chagué et al. 2002).

In plants, ethylene is synthesized from methionine through two intermediates, S-adenosyl-methionine (S-AdoMet) and 1-amino-cyclopropane-1-carboxylic acid (ACC) (Adams and Yang 1979). In microorganisms, ethylene biosynthesis can occur via three

pathways, one of which is the pathway employed by plants (Jia et al. 1999). Another pathway involves a multifunctional enzyme called ethylene-forming enzyme (EFE), which converts 2-oxoglutarate (OXO) to ethylene (Fukuda et al. 1986; Hottiger and Boller 1991; Chagué 2010). Ethylene can also be produced via oxidation of α-keto-γ-methylthiobutyric acid (KMBA), a substrate synthesized through deamination of methionine (Primrose 1977; Chagué et al. 2002; Cristescu et al. 2002). The ethylene biosynthesis pathway of *V. dahliae* was characterized by Tzeng and DeVay (1984) who showed that two isolates from cotton could produce ethylene via the KMBA pathway and that addition of ACC to culture medium inhibited ethylene production. The genome of *V. dahliae* (www.broadinstitute.org) contains genes encoding ACC synthase, ACC deaminase, and EFE. However, to date their role in pathogenicity and ethylene biosynthesis has not been studied.

Many plant growth promoting rhizobacteria (PGPR) that produce ACC deaminase, cleave ACC into α-ketobutyrate and ammonia and promote plant growth presumably by lowering plant ethylene levels (Honma and Shimomura 1978; Glick et al. 1998). It has been shown that treatment of plants with ACC deaminase-producing PGPR can provide significant protection to plants against several phytopathogenic fungi as *Pythium ultimum* (Wang et al. 2000), *Sclerotium rolfsii, Rhizoctonia solani* (Husen et al. 2011), and *Pyricularia oryzae* (Amutharaj et al. 2012). However, to the best of our knowledge, the role of *ACC deaminase* gene in virulence and ethylene production, has never been studied in plant pathogenic fungi.

The aim of this study was to investigate if and how ACC deaminase affects the pathogenicity of *V. dahliae* and its ethylene production by disrupting the gene encoding this enzyme, entitled *ACCd*, and also overexpressing it. The resulting transformed strains were evaluated for their virulence under different plant growth conditions, their morphological characteristics and their ability to produce ethylene. It was shown that the *ACCd* overexpressing mutants resulted in increased virulence in tomato and eggplant while deletion mutants exhibited less severe symptoms in inoculated plants. This phenotype was not associated with ethylene production since both types of mutant produced more ethylene than the wild type strain (70V-WT). However *ACCd* mutant differed in ACC content, thus indicating that accumulation or reduction of ACC in the roots may be involved in virulence rather than the production of ethylene by the mutants.

Materials and Methods

Fungal isolates and culture conditions

Strain 70V-WT, a *Verticillium dahliae* race 1 isolate (Pantelides et al. 2010b) and Vd-K, a *V. dahliae* race 2 isolate (Dobinson 1995) isolated both from tomato, were used throughout this study. For long term storage, these isolates and their transformants were preserved as a conidial suspension (4 x 10^7 conidia ml^{-1}) in 25 % glycerol at -80°C. They were activated by culturing on PDA (Potato Dextrose Agar, OXOID) for 5 days at 25°C. Transformants were cultured on medium supplemented with 50 μg ml^{-1} geneticin (Life Technologies) or hygromycin B (Duchefa Biochemies) to maintain pure culture of fungal cells expressing the plasmid constructs used for transformation.

Minimal medium (Puhalla and Mayfield 1974) was used for mycelial growth. Conidial production was assessed under two culture conditions at 25°C: (a) PDA for 20 days and (b) shaking in liquid Sucrose Sodium Nitrate (SSN) medium for 7 days (Sinha and Wood 1968) using an orbital incubator at 140 rpm. For observation of spore germination and plant infection, spores produced in SSN medium were used. Ethylene production was measured after culturing at 25°C in liquid Czapek-Dox medium (OXOID) supplemented with 10mM methionine (Sigma-Aldrich) or 10 mM 2-oxoglutarate (α-ketoglutaric acid sodium salt, Sigma-Aldrich) by shaking at 140 rpm.

Nucleic acid manipulation

Fungal genomic DNA was extracted as previously reported (Cary et al. 2009) with a few modifications. After culturing on PDA for 7 days at 25°C, mycelia were scraped off and placed in 1.5 ml tube. The mycelia were macerated using a mini plastic pestle after adding 700 μl of LETS buffer (0.1 M LiCl, 20 mM EDTA, 10 mM Tris-HCl), mixed by inversion and incubated at room temperature for 5 min. After adding 700 μl of phenol:chloroform:isoamyl alcohol (25:24:1), it was mixed by inversion and centrifuged at 16000 g at 4°C for 15 min. The supernatant was transferred to a new tube, and 1 ml of 95% ethanol was added. Nucleic acids were precipitated by centrifugation at 16000 *g* for 10 min at room temperature, washed with 500 μl of 70% ethanol, dried at room temperature for 5 min, and resuspended in 30 μl sterile distilled water. For quantification of fungal biomass in plant stems, total genomic DNA was isolated as described in Dellaporta et al. (1983).

DNA amplification for gene manipulation was performed using KAPA HiFi DNA polymerase (KAPA Biosystems). PCR conditions were: one cycle of 95°C for 3 min; 35 cycles of 98°C for 20 sec, 72°C for 15 sec, and 72°C for 30 sec; one cycle of 72°C for 1 min. Screening of transformants was done by PCR using KAPA polymerase (KAPA Biosystems) and the conditions used were: one cycle of 95°C for 3 min; 35 cycles of 95°C for 30 sec; 60°C for 45 sec; 72°C for 3 min; one cycle of 72°C for 1 min. *Escherichia coli* strain DH5a was used for plasmid propagation. Plasmid DNA isolation, digestion with restriction enzymes, ligation, and *E. coli* transformation were performed as described in Sambrook et al. (1989).

Total RNA was extracted from mycelia ground in the presence of liquid nitrogen using RNAiso plus reagent (TAKARA). RNA samples were treated with DNase (HT Biotechnology) to eliminate DNA contamination. First-strand cDNA was synthesized using PrimeScript RT reagent kit (TAKARA).

Vector construction and fungal transformation

A construct for overexpressing *ACCd* was prepared as outlined in Fig. 1. Primers ACD_OX_F and ACD_OS_R (Supplementary Table 1) were used to amplify the ORF of *ACCd* gene (1038 bp). The ACD_OX_F and ACD_OS_R primers contained the *Xba*I and *Sph*I restriction sites at the 5' end, respectively, to clone the resulting PCR product between the *Magnaporthe oryzae* ribosomal protein promoter and the *Neurospora crassa* β-tubulin terminator in vector pSK1226 and the *Aspergillus nidulans* TrpC promoter and the *Neurospora crassa* β-tubulin terminator in vector pSK1727. The resulting constructs (1835 and 1679 bp) were cloned between the *Eco*RI and *Hin*dIII sites of binary vector pSK879, which carries the geneticin resistance cassette (Fig. 1). The resulting plasmids, termed as p1226VdAD and p1727VdAD, were introduced into *Agrobacterium tumefaciens* strain AGL1 for fungal transformation. *Agrobacterium tumefaciens*–mediated transformation (ATMT) was performed as described by Mullins et al. (2001), with the following modifications: i) Co-cultivation of *V. dahliae* spores and *A. tumefaciens* cells was performed on positively charged nylon membrane (Porablot NY plus, Macherey-Nagel); ii) PDA amended with 50 μg ml^{-1} geneticin or hygromycin B, 100 μg ml^{-1} moxalactam, and 200 μM cefotaxime was used to select transformants. Transformants were purified via single-spore isolation.

A mutant allele for *ACCd* disruption was constructed as summarized in Fig. 2A. Primer pairs ACD_EX-F1/ACD_K-R1 and ACD_X-F2/ACD_H-R2 (Supplementary Table 1) were used to amplify the putative promoter (827 bp) and terminator (864 bp) regions of the gene, respectively from 70V-WT genomic DNA. The amplified regions were cloned into the 5’ and 3’ end of the geneticin resistance cassette in vector pSK666 (Tzima et al. 2010). The resulting mutant allele (3171 bp) was amplified with primers ACD-P_F1 and ACD_H-R2 and cloned between the *Pst*I and *Hin*dIII sites of binary vector pGKO2 (Khang et al. 2005), which carries the HSVtk (herpes simplex virus thymidine kinase) gene as a negative selection marker against ectopic transformants. The resulting plasmid pGVdAD in *A. tumefaciens* AGL1 was used for disrupting *ACCd*. Transformants were selected as described above except that 50 μM 5-fluoro-2-deoxyuridine (F2dU; Sigma-Aldrich) was used to select against ectopic transformants (Khang et al. 2005).

For complementation, a fragment of 2728 bp, which covers the *ACCd* gene including 1000 bp 5’ and 600 bp 3’ regions of the ORF, was amplified from 70V-WT using primers ACD_X-C-F1 and ACD_H-R2 (Supplementary Table 1). The resulting PCR product was cloned between the *Xba*I and *Hin*dIII sites of binary vector pSK561 (Tzima et al. 2010b), which carries a hygromycin resistance gene cassette.

Molecular characterization of resulting mutants

Initial screening for the identification of the deletion mutant and complemented strains was performed by PCR using primers ACD_EX-F1 and ACD_H-R2 (Supplementary Table 1). *ACCd* transcripts in these mutants were quantified using reverse transcription PCR analysis (RT-PCR) with ACD_OX_F and ACD_OS_R primers on cDNAs. Transcripts in strains that overexpress *ACCd* were measured by quantitative real-time reverse transcription PCR analysis (qRT-PCR) as described below.

Growth and developmental characteristics of resulting mutants

Deletion mutants and overexpression strains derived from 70V-WT were characterized to determine their developmental and morphological traits. For each strain, 20 μl conidial suspension ($1x10^6$ conidia ml^{-1}) was spot inoculated on five minimal medium plates, containing 20 ml of medium per plate, and incubated at 25°C.

Colony diameters and morphology were examined at 8, 10, 12, 14, 16 and 21 days post-inoculation (dpi). For measuring conidial production, individual isolates were grown on PDA, containing 20 ml of medium per plate, at 25°C for 20 days (three plates per strain). After adding 4 ml of sterilized water to each plate, the surface was rubbed using a sterile swab and plates were shaken gently to release conidia. A hemocytometer was used to count the number of conidia in 20 μl conidial suspension under a light microscope (Axiostar plus, Carl Zeiss International, Germany). The concentration was expressed as conidia per cm^2 of colony.

Conidial production in liquid SSN medium was quantified by inoculating 300 μl spore suspension ($1x10^6$ conidia ml^{-1}) for each strain into 100 ml medium in 250 ml Erlenmeyer flask (three flasks for each isolate) and shaking (150 rpm) at 25°C. After 7 days, the number of spores in 20 μl medium were counted as described above. For germination test, 10 μl spore suspension ($1x10^6$ conidia ml^{-1}) of individual strains was placed on sterile glass slides (three slides for each isolate). Germination rate was determined by observing approximately 100 conidia per slide after 12, 16 and 20 hours of incubation at 25°C under 100% humidity. To examine the effect of ACC on radial growth and spore production, individual strains were grown on supplemented with 0, 10, 100 and 500 μM ACC. Their colony diameters were determined 17 days after inoculation. Spore production was assayed as described above.

Conditions for plant growth and infection

Seeds of tomato (*Solanum lycopersicum* cv Ailsa Craig) and eggplant (*Solanum melongena* cv Black Beauty) were stored at 4°C. For infection assays in growth room, after initially growing seedlings in plastic tray, they were transplanted to 10.5 cm-diameter pot (Teku, VCG 10.5 pot) with each pot containing approximately 450 ml soil (Plantaflor Potting Soil, Germany). These pots were placed in a growth room set at 65–70% RH, 25°C, and a 16-h photoperiod with a photon flux density of 200 ± 20 μmol m^{-2} s^{-1}. Plants were watered every two days to maintain 70% soil humidity. For infection assays in greenhouse, seeds were sown directly in the same pots, and plants were watered as needed.

Five day-old fungal culture in SSN medium was passed through cheesecloth to remove mycelia, and the conidial concentration was adjusted to $1x10^7$ conidia ml^{-1}.

Each seedling was inoculated with 10 ml conidia suspension via root drenching when they reached the four-leaf stage (tomato) or two-leaf stage (eggplant).

Disease severity was measured based on the number of wilted leaves as a percentage of the total number of leaves for each plant. Symptoms were recorded for up to 19 days and plotted over time to generate the area under the disease progress curve (AUDPC) using the trapezoidal integration method (Campbell and Madden 1990). Disease was expressed as a percentage of the maximum possible area for the whole period of the experiment, referred to as relative AUDPC. Plant height and fresh weight were also measured. The experiments were repeated two times with 12 (growth room) and 5 (greenhouse) plants per experiment for tomato and 10 plants per experiment for eggplant.

Hydroponically grown plants were also infected to microscopically observe root attachment and colonization. Tomato seedlings grown in plastic trays were transplanted to 1 L container (1 plant per container) containing complete nutrient solution at the first leaf stage. Containers were covered with aluminium foil (Fig. 9) to prevent algal growth and temperature fluctuation. Plants were grown for two weeks to allow recovery from transplanting stress. Complete nutrient solution consisted of the following minerals: NO_3^- = 14.29, K = 8.43, PO_4^{-3} = 1.07, Ca = 6.96, Mg = 2.93, SO_4^{-2} = 1.56, Na = 1.91 mmol L^{-1} respectively , and B = 25.00, Fe = 20.85, Mn = 10.00, Cu = 0.77, Zn = 3.85, and Mo = 0.52 µmol L^{-1}, respectively (Marinou et al. 2013). At the end of the 2nd week, six plants were inoculated with conidia at the final concentration of $1x10^6$ conidia ml^{-1}. Additional nutrient solution was added weekly. The pH and electric conductivity of the solution were maintained at 5.8 and 2.1 mS cm^{-1}, respectively. Due to the alkalinity of water used, the pH was measured every third day and adjusted if needed using H_2SO_4 (5% v/v). Symptoms were recorded at 14 and 17dpi.

Quantification of fungal biomass *in planta*

Fungal biomass in infected plants was quantified by qPCR. For each biological sample (pool of three plants per treatment), the above-ground parts of inoculated plants were collected and ground to a fine powder using an autoclaved mortar and pestle in the presence of liquid nitrogen. DNA was extracted as described above, quantified using Nano Drop 2000 spectrophotometer (Thermo Scientific), and adjusted to 100 ng µl^{-1}. qPCR reactions were performed using IQ5 thermocycler (Bio-Rad) and the KAPA

SYBR FAST qPCR kit (KAPA Biosystems). Primers ITS1-F and ST-VE1-R were used for quantifying *V. dahliae*, the *S. lycopersicum β-tubulin* gene and the *S. melongena* actin gene were used to normalize the amount of fungal DNA in different samples (Supplementary Table 1).

Microscopic observation of root infection

To compare root attachment and colonization during early stages of infection between 70V-WT and its mutants, hydroponically grown tomato plants were infected with *70V-WTR*, *Vd6ADM1R*, and *70ΔAD3.4R*, transformants of 70V-WT, *Vd6ADM1*, and *70ΔAD3.4* with the *AsRed* gene under the control of the *F. verticillioides* elongation factor 1α promoter (Kim et al. 2011). At 1 and 5 dpi, plants were removed, washed with distilled water, and the root surface was imaged using a Zeiss Axio Imager D1 epi-fluorescence microscope equipped with a Zeiss AXIO Cam MRc5 camera (Carl Zeiss International, Germany). Filter sets used for observing AsRed were 576 nm (excitation) and 592 nm (emission). Collected images were stored as jpeg files and processed using Adobe Photoshop CS software (Adobe Systems Inc., USA).

Ethylene Production

To determine whether disruption or overexpression of *ACCd* gene affects ethylene production, wild type strain 70V-WT and its transformants were evaluated for their ability to produce ethylene. Individual strains were cultured in 50 ml Erlenmeyer flask containing 20 ml Czapek Dox broth supplemented with 10 mM methionine and 50 μg ml-1 geneticin or hygromycin or 10 mM 2-oxoglutarate. Each flask was inoculated with 10^6 conidia and was placed in an orbital shaker (150 rpm) set at 25°C. Before ethylene measurement, flasks were flushed with air to eliminate any accumulated ethylene. After sealing with rubber stopper for 1 hour, gas samples were collected from the headspace using a gas-tight syringe. The amount of ethylene in 1 ml gas sample was measured using 7890A Agilent gas chromatographer (Agilent Technologies), equipped with a flame ionization detector and an Agilent stainless steel column (2mm id x 2m) packed with Porapak Q 80/100 mesh, as described by Petrou et al. (2013) with small modifications. Helium was used as carrier gas under a constant inlet pressure of 20 psi. Inlet and oven temperatures were maintained at 80 °C, and detector temperature was set at 200°C. The total runtime was 3 min with ethylene being detected at 1.8 min.

Calibration was performed using 1 ppm ethylene. After ethylene measurement, the mycelia and conidia in each flask were harvested by centrifugation and were lyophilized to determine dry weight. Ethylene production rate was calculated as nanoliters of ethylene produced per hour per gram of dry mycelium (nl h^{-1} g^{-1}) or nanoliters of ethylene per culture. The experiment was repeated twice and the data shown corresponded to the averages of three independent biological samples.

ACC measurement in 70V-WT and *ACCd* mutants

For quantifying the amount of ACC in *V. dahliae* 70V-WT and its *ACCd* transformants, fungal strains were cultured in liquid SSN medium at 25°C by shaking (140 rpm) for 7 days, and harvested spores and mycelia were freeze-dried for 48 hours. All samples were immediately frozen by immersing them in liquid nitrogen before freeze-drying. The amount of ACC was measured using the method described in Lizada and Yang (1979) in which the amount of ACC formed was measured via its conversion to ethylene. Ethylene was determined by gas chromatography as described above. Fungal ACC content shown correspond to the averages of three independent biological samples.

KMBA detection

Production of KMBA by 70V-WT and its transformants was determined by precipitation with 2, 4-dinitrophenylhydrazine according to Primrose (1977). The wild type 70V-WT and the mutant strains were grown in liquid Czapek Dox and the culture medium was separated from the mycelium by filtration. Subsequently, 1 ml of 0.1% 2, 4-dinitrophenylhydrazine in 2 M HCl was added to 10 ml of culture filtrate and stirred at room temperature in total darkness. 10 mM solution of KMBA (α-keto-γ-methylthiobutyric acid sodium salt, Sigma-Aldrich) in Czapek-Dox was used as a positive control. In the presence of KMBA a yellow precipitate was formed after 30 min.

Gene expression analysis

To examine transcription levels of *ACCd* gene and genes involved in ethylene biosynthesis, qRT-PCR was performed on cDNAs of 70V-WT and its transformants. Primers used in gene expression analysis are listed in Supplememtary Table 1. The *V.*

dahliae β-tubulin gene was used as an internal standard to normalize small differences in cDNA template amounts. For data analysis, average threshold cycle (Ct) values were calculated for each gene of interest (Pfaffl 2001) on the basis of three independent biological samples.

Statistical analysis

Statistical analyses were performed using R package (R 2016, https://www.r-project.org, version 3.3.1). Data obtained were subjected to analysis of variance (ANOVA) and significant differences between individual means were then determined using Tukey's post hoc pairwise comparison test at the 5% confidence level. Dunnett's multiple comparisons test was used for analyzing gene expression data from *ACCd* overexpression mutants and 70V-WT.

Results

Overexpression and disruption of the *ACCd* gene

The *ACCd* gene of the soil-borne fungus *V. dahliae* is a single-copy gene with a coding sequence of 1128 bp that contains an open reading frame (ORF) of 1038 bp, interrupted by one intron of 90 bp long, located at position 186 and is predicted to encode a protein of 345 amino acids. Its ORF driven by two strong constitutive promoters, the *Magnaporthe oryzae* ribosomal protein promoter and the *TrpC* promoter of the fungus *Aspergillus nidulans,* was introduced in strains 70V-WT and Vd-K via *Agrobacterium tumefaciens* mediated transformation (ATMT) (Fig. 1A). Twelve transformed strains per each promoter per wild type isolate were evaluated by qRT-PCR for their ability to overexpress the *ACCd* gene (Figs. 1B and 1C, Supplementary Fig. 1). Three transformants of the wild type strain 70V-WT (*Vd6ADM1, Vd6ADM2* and *Vd6ADM10*), that overexpressed the *ACCd* gene under the control of the *Magnaporthe oryzae* ribosomal protein promoter, showed up to 12 times higher expression levels compared to the wild type strain and were chosen for subsequent experiments (Fig. 1B).

The *ACCd* gene was also deleted (Fig. 2A). Thirty-four out of thirty-eight geneticin-resistant transformants of 70V-WT were desired mutants as revealed by PCR (Supplementary Fig. 2). The absence of *ACCd* transcripts in two mutants (*70ΔAD1.4* and *70ΔAD3.4*) was also confirmed by RT-PCR analysis (Fig. 2C). A functional copy of *ACCd*, including promoter and terminator sequences, was successfully reintroduced in mutant *70ΔAD1.4* for complementation resulting in the complemented deletion strain *70CAD1.2.2* (Fig. 2B). The amount of *ACCd* transcripts in *70CAD1.2.2* was comparable to that in 70V-WT (Fig. 2C).

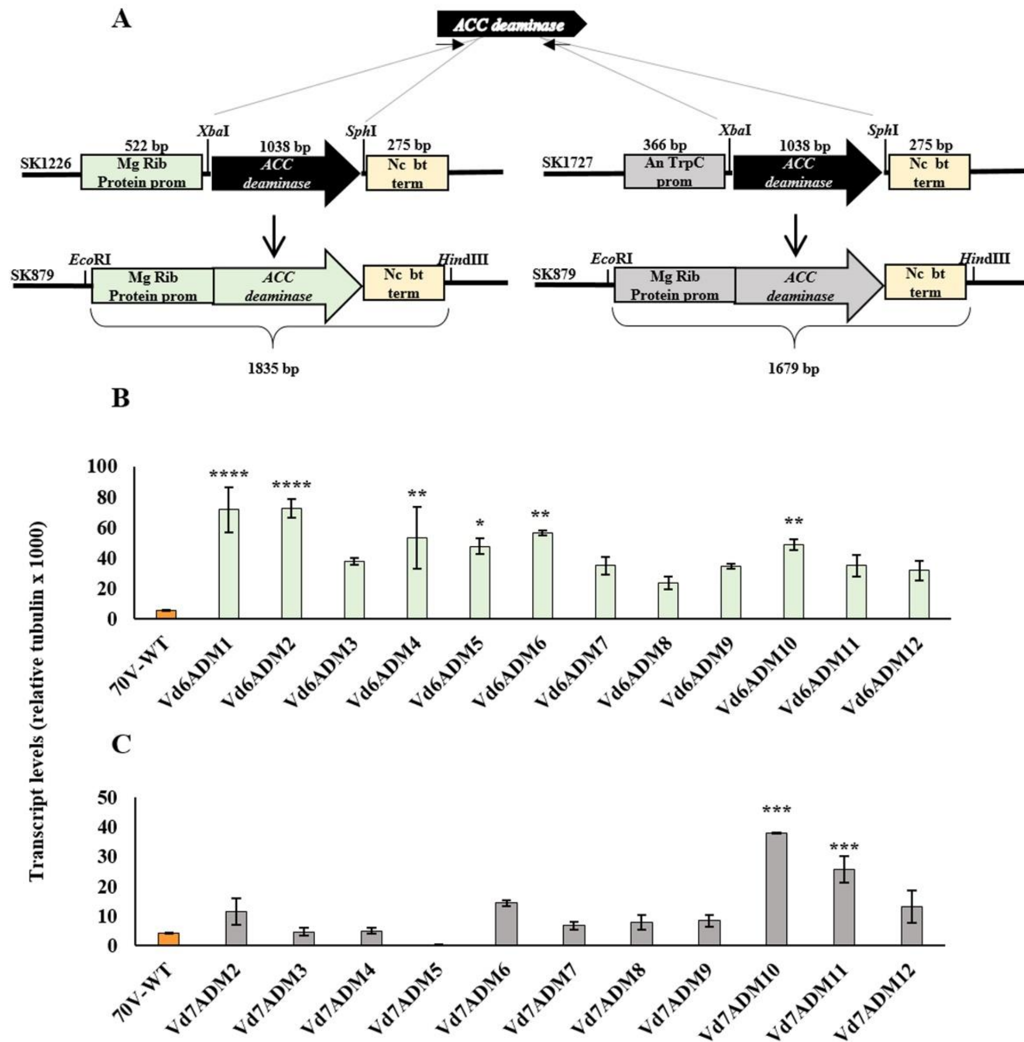


Fig. 1. Overexpression of *ACCd* in *V. dahliae*. (A) The overexpression constructs, consisting of the *ACCd* ORF under the control of the *M. oryzae* ribosomal protein promoter and *N. crassa* β-tubulin (bt) terminator (1835 bp) and the *A. nidulans TrpC* promoter and the *N. crassa* β-tublin (bt) terminator (1679 bp), were cloned in pSK879, a binary vector for *Agrobacterium tumefaciens* mediated transformation. Levels of *ACCd* transcripts in mutants overexpressing the *ACCd* gene under **(B)** the *M. oryzae* ribosomal protein promoter and **(C)** *A. nidulans TrpC* promoter were measured using qRT-PCR. Vertical bars indicate standard errors calculated using data from three replicates. Columns with asterisks indicate statistically significant differences between each mutant and 70V-WT according to Dunnett's multiple comparison test (*$P \leq 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

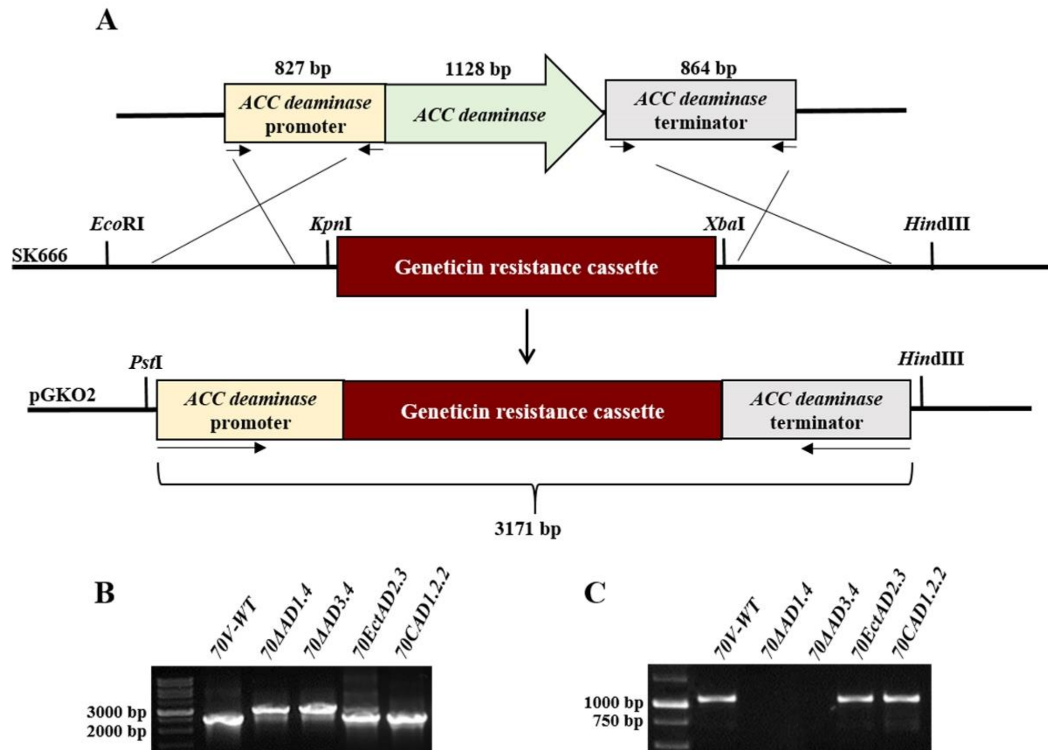


Fig. 2. **Deletion of the *ACCd* gene in *V. dahliae*.** **(A)** Construction of a mutant allele for *ACCd* deletion. Middle line shows cloning of the putative *ACCd* promoter and terminator regions in the geneticin resistance vector SK666. Bottom line illustrates cloning of the mutant allele (3171bp) in binary vector pGKO2 for ATMT fungal transformation. The location and orientation of the primers used for constructing the mutant allele are noted by black arrows **(B)** PCR confirmation of *ACCd* disruption. Amplification of the 3171 bp fragment of the *ACCd* mutant allele in mutants *70ΔAD1.4* and *70ΔAD3.4*. In wild type strain 70V-WT, ectopic transformant *70EctAD2.3* and the complemented deletion mutant *70CAD1.2.2* a 2728 bp product of the *ACCd* gene was amplified. **(C)** Absence of *ACCd* transcripts in *70ΔAD1.4* and *70ΔAD3.4* was confirmed via reverse transcription PCR.

Effect of both mutations on growth, microsclerotia formation, and conidial production and germination

70V-WT and its mutants were cultured on minimal medium to investigate the role of *ACCd* in growth and microsclerotia production. Their colony sizes were similar (Fig. 3A). However, compared to 70V-WT, which formed abundant darkly pigmented microsclerotia, both types of mutants produced less microsclerotia but more aerial hyphae (Figs 3C and 3D).

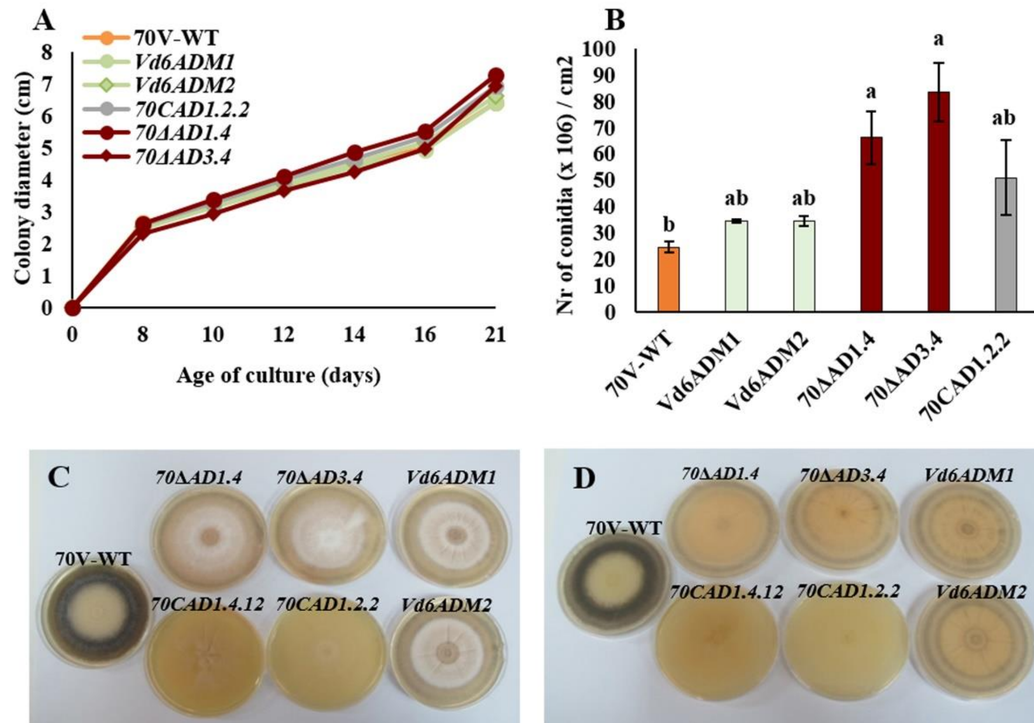


Fig. 3. Colony growth, microsclerotia formation and spore production of 70V-WT and *ACCd* mutants. (**A**) Colony growth over time and (**B**) spore production of 70V-WT, *ACCd* deletion (*70ΔAD1.4* and *70ΔAD3.4*) and overexpression (*Vd6ADM1* and *Vd6ADM2*) mutants and a complemented deletion mutant (*70CAD1.2.2*) on PDA. Vertical bars in **A** and **B** indicate the calculated standard errors of five and three replicates, respectively. Different letters in **B** note statistically significant differences according to Tukey's multiple range test at $P \leq 0.05$. Colony appearance 30 days after inoculation from the (**C**) top and (**D**) bottom of representative plates is shown.

When cultured on PDA, deletion mutants *70ΔAD1.4* and *70ΔAD3.4* produced significantly more conidia than 70V-WT (Fig. 3B), but no significant difference was noted in liquid SSN medium (Supplementary Fig. 3A). However, on PDA amended with 10 or 100 µM ACC, significantly reduced conidial production was observed in *70ΔAD3.4* (Fig. 4A). Conidial production by overexpression mutants *Vd6ADM1* and *Vd6ADM2* and complemented deletion mutant *70CAD1.2.2* was comparable to 70V-WT when cultured on PDA (Fig. 3B), in SSN (Supplementary Fig. 3A) and on PDA supplemented with ACC (Fig. 4B). ACC at 500 µM appeared inhibitory to colony growth of 70V-WT, *Vd6ADM1* and *70CAD1.2.2* strains (Fig. 4A). Spore germination was similar among all strains (Supplementary Fig. 3B).

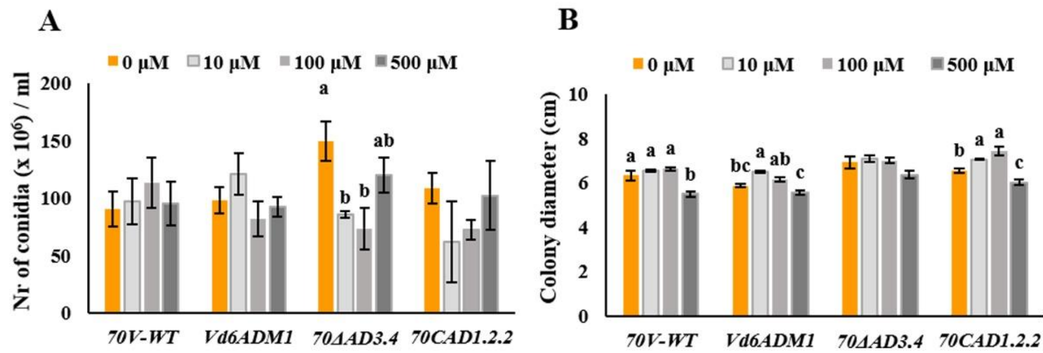


Fig. 4. Colony diameter and spore production of 70V-WT and *ACCd* mutants in the presence of ACC. 70V-WT, deletion mutant *70ΔAD3.4*, overexpression mutant *Vd6ADM1*, and complemented deletion mutant *70CAD1.2.2* were cultured on PDA supplemented with 0, 10, 100 or 500 µM ACC. Their **(A)** colony diameter and **(B)** spore production were measured. Vertical bars indicate the calculated standard errors of **(A)** five and **(B)** three replicates. All values were subjected to analysis of variance. For data that showed a significance level of $P \leq 0.05$, Tukey's post hoc analysis was also performed.

Involvement of *ACCd* in pathogenesis

Virulence of deletion and overexpression mutants in tomato and eggplant was evaluated. Infection of tomato plants was performed in both growth room and greenhouse. Compared to 70V-WT, two deletion mutants caused significantly less disease, whereas plants infected with two overexpression strains displayed more severe wilting symptoms (Fig. 5A). Tomato plants inoculated with 70V-WT and overexpression mutants *Vd6ADM1* and *Vd6ADM2* exhibited the first wilting symptoms at 8 days post inoculation (dpi). At 14 dpi, the disease severity caused by *Vd6ADM1* and *Vd6ADM2* reached 50% and 42%, respectively, while that caused by 70V-WT reached 25%. Plants inoculated with deletion mutants *70ΔAD1.4* and *70ΔAD3.4* displayed only mild chlorosis in older leaves at 14 dpi with disease severity being 12% and 12.5%, respectively (Fig. 5B). Consistent with the onset and severity of symptom development, the relative AUDPC values showed that *70ΔAD1.4* and *70ΔAD3.4* (7%) caused significantly less disease than 70V-WT (13.9%) and complemented deletion mutant *70CAD1.2.2* (10.3%). Plants inoculated with *Vd6ADM1* and *Vd6ADM2* showed relative AUDPC values of 31.3% and 26.4%, respectively (Fig. 5C). Compared to plants inoculated with 70V-WT, average heights and fresh weights of plants inoculated with *70ΔAD1.4* and *70ΔAD3.4* were higher, while those in plants inoculated with *Vd6ADM1* and *Vd6ADM2* were significantly lower (Figs. 5D and 5E). Although the difference between 70V-WT and two overexpression mutants was less pronounced in greenhouse

evaluations, similar patterns were observed (Supplementary Fig. 6). The amounts of disease expressed as relative AUDPC after infection by *Vd6ADM1* and *Vd6ADM2* were 17% and 20%, respectively, while 70V-WT, *70ΔAD1.4*, and *70ΔAD3.4* resulted in 13.6%, 7.6%, and 9.8%, respectively.

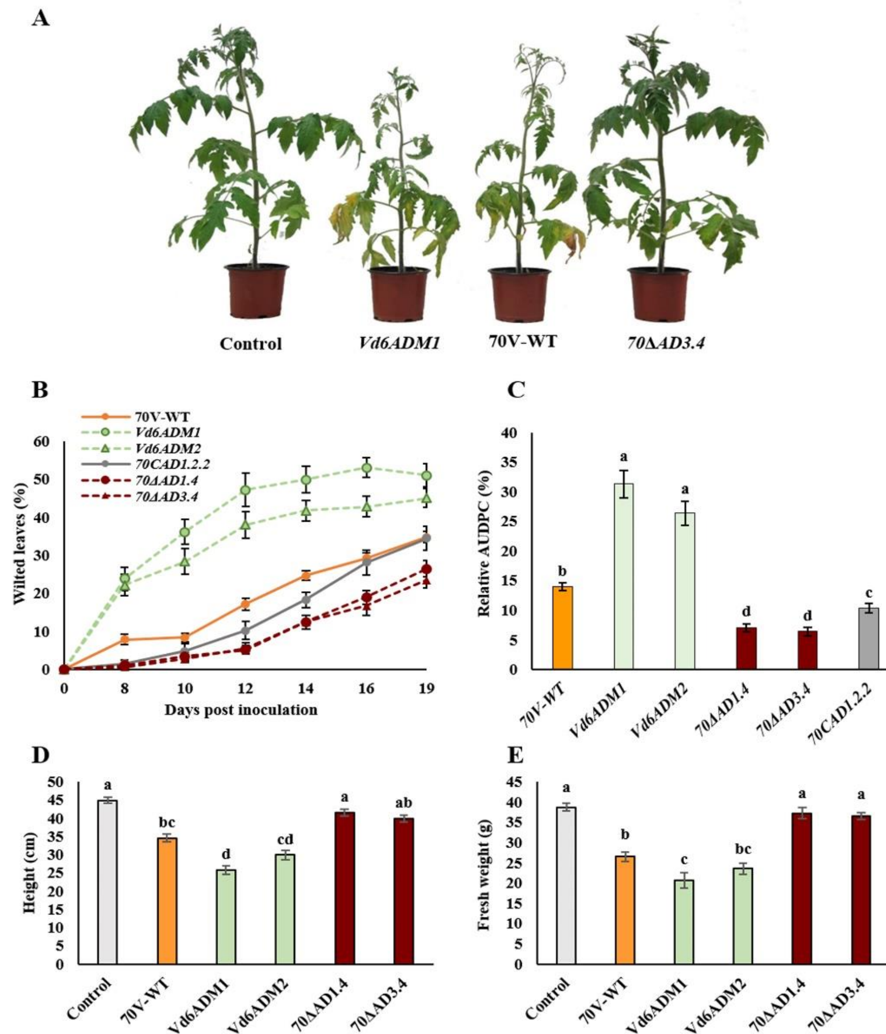


Fig. 5. Disease severity of tomato plants infected with 70V-WT and *ACCd* mutants in growth room. Tomato plants were inoculated with 70V-WT, deletion (*70ΔAD1.4* and *70ΔAD3.4*) and overexpression (*Vd6ADM1* and *Vd6ADM2*) mutants, and complemented deletion mutant *70CAD1.2.2*. **(A)** Representative tomato plants at 19 days after inoculation are shown. **(B)** Disease progress over time, **(C)** the amount of disease expressed as relative AUDPC, and the **(D)** height and **(E)** fresh weight of plants, are shown. Vertical bars indicate the standard errors based on twenty-four replicates. All values were subjected to analysis of variance. Different letters in **C**, **D** and **E** note statistically significant differences according to Tukey's multiple range test at $P \leq 0.05$.

Fungal biomass in the vascular tissues of infected plants was assessed using qPCR to determine whether it correlates with disease severity. At 8 dpi, fungal DNA was detectable in all treatments. However, the amount of fungal DNA in plants infected with *Vd6ADM1* and *Vd6ADM2* was significantly greater than that in plants infected with 70V-WT (Fig. 6A). The amount of DNA for deletion mutants *70ΔAD1.4* and *70ΔAD3.4* was similar to that of 70V-WT (Fig. 6A). At 21 dpi, the amount of DNA for *Vd6ADM1* and *Vd6ADM2* was 20-fold and 11-fold greater than that of 70V-WT. However, the difference between 70V-WT and deletion mutants was not significant (Fig. 4B).

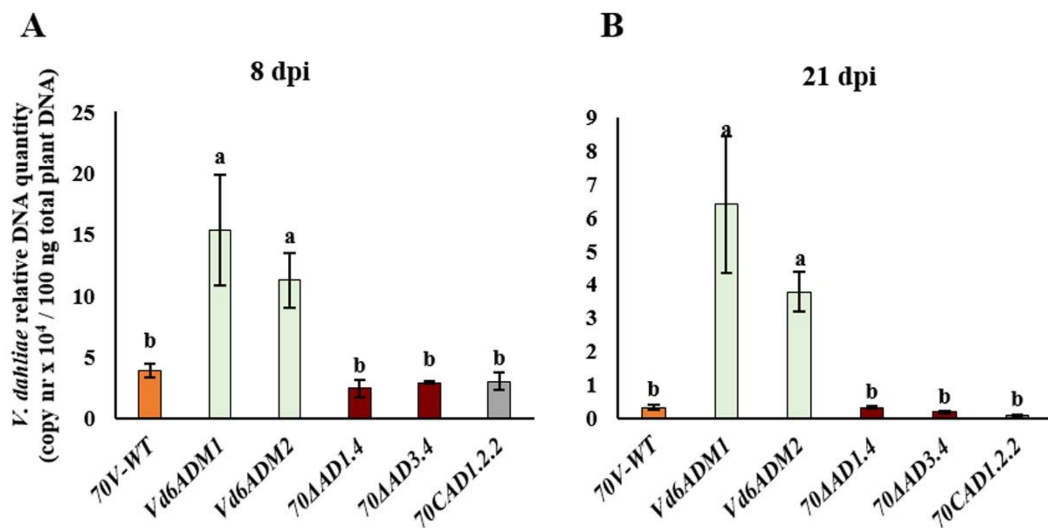


Fig. 6. Quantification of fungal biomass in infected plants. The amount of fungal DNA in the stem of tomato plants was quantified at **(A)** 8 and **(B)** 21 days after inoculation with 70V-WT, deletion (*70ΔAD1.4* and *70ΔAD3.4*) and overexpression (*Vd6ADM1* and *Vd6ADM2*) mutants, and complemented deletion mutant *70CAD1.2.2*. Vertical bars indicate the calculated standard errors of three replicates (pool of three plants per replicate per treatment). Columns with different letters were statistically significantly different according to Tukey's multiple range test at $P \leq 0.05$.

In eggplant inoculated with three overexpression mutants, disease progressed more rapidly, causing severe wilting symptoms and leaf abscission (Fig. 7A). At 14 dpi, about 60% of the leaves of plants infected with *Vd6ADM1, Vd6ADM2*, and *Vd6ADM10* displayed wilt symptoms, while ~50% of the leaves showed wilt symptoms in plants infected with 70V-WT (Fig. 7B). Relative AUDPC values were consistent with higher virulence of *Vd6ADM1, Vd6ADM2*, and *Vd6ADM10* (35%, 33%, and 37%,

respectively) than that of 70V-WT (23%) (Fig. 7C). Fungal DNA was detected in all treatments at 12 dpi. The amount of fungal DNA in plants infected with overexpression strain *VdADM10* was significantly greater than that in plants infected with 70V-WT at 12dpi, but no significant difference was observed among the rest of the strains at both time points of observation (Figs. 7D and 7E).

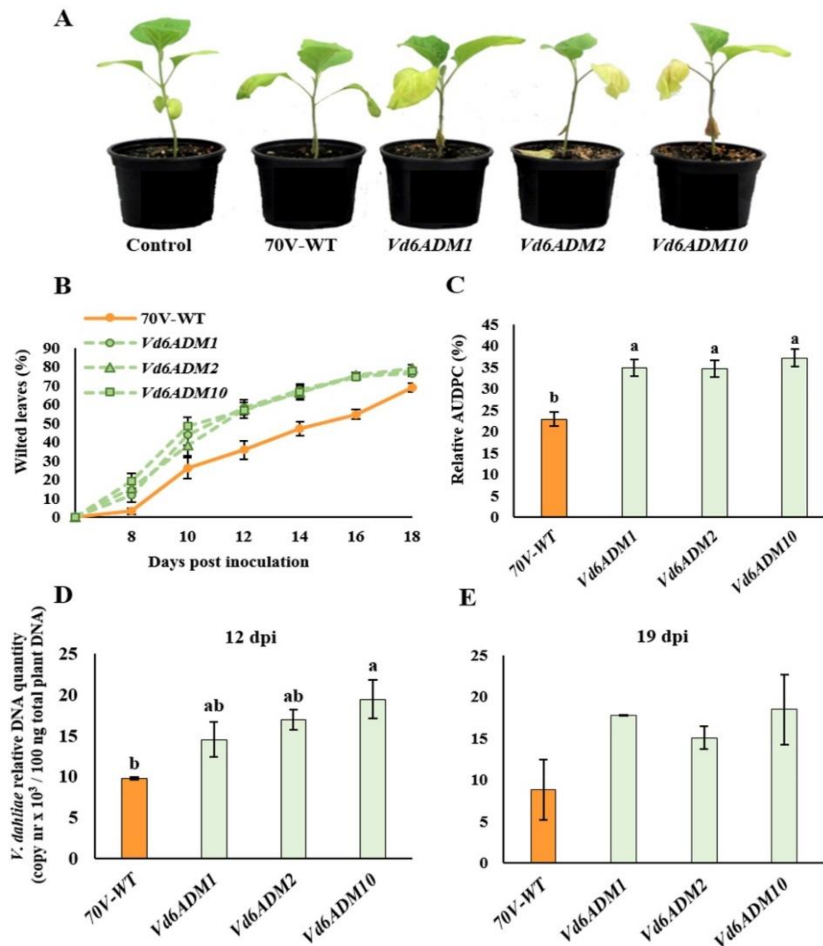


Fig. 7. Disease symptoms in eggplant infected with 70V-WT and *ACCd* overexpression mutants in growth room. Eggplant was infected with 70V-WT and three overexpression mutants (*Vd6ADM1, Vd6ADM2* and *Vd6ADM10*). **(A)** Symptoms at 14 days after inoculation, **(B)** disease progression over time, and **(C)** relative AUDPC are shown. Vertical bars indicate the standard errors based on twenty replicates. Amount of fungal DNA in the stem of plants was quantified at **(D)** 12 and **(E)** 19 days after inoculation with 70V-WT and overexpression mutants. Vertical bars indicate the calculated standard errors of three replicates (pool of three plants per replicate per treatment). All values were subjected to analysis of variance. Different letters in **C and D** note statistically significant differences according to Tukey's multiple range test at $P \leq 0.05$.

Similar to tomato, eggplant infected with deletion mutants showed less chlorosis and wilting symptoms (Fig. 8A) with disease severity at 13 dpi being 40% and 33%, respectively compared to 60% caused by 70V-WT (Fig. 8B). Relative AUDPC values after infection by *70ΔAD1.4* and *70ΔAD3.4* were 23% and 19%, respectively, while that of 70V-WT was 33% (Fig. 8C).

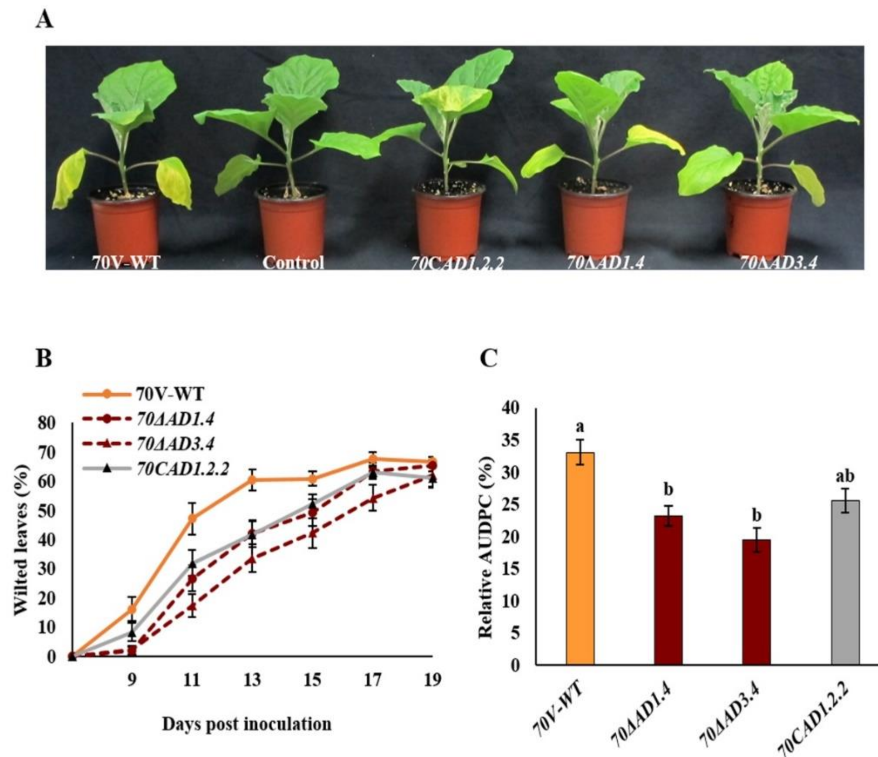


Fig. 8. Disease symptoms in eggplant infected with 70V-WT and *ACCd* deletion mutants in growth room. Eggplant was infected with 70V-WT, two deletion mutants (*70ΔAD1.4* and *70ΔAD3.4*), and complemented deletion mutant *70CAD1.2.2*. **(A)** Symptoms at 13 days after inoculation, **(B)** disease progression over time, and **(C)** relative AUDPC are shown. Vertical bars indicate the standard errors based on twenty replicates. All values were subjected to analysis of variance. Different letters in **C** note statistically significant differences according to Tukey's multiple range test at $P \leq 0.05$.

ACCd manipulations did not affect conidial adhesion to tomato roots

To facilitate monitoring of conidial attachment to roots and early stages of root colonization, deletion mutants *70ΔAD1.4* and *70ΔAD3.4*, overexpression mutant *Vd6ADM1*, and 70V-WT were transformed to constitutively express a red fluorescent protein (AsRed), resulting in *70ΔAD1.4R, 70ΔAD3.4R*, *Vd6ADM1R*, and 70V-WTR. At 1 dpi, conidia of all strains attached to the root surface similarly (Fig. 9A-D). At 5 dpi,

no significant differences in spore attachment and root colonization were observed (Fig. 9E-H). However at 17 dpi strain *Vd6ADM1R* had colonized the vascular tissues of the plants in a greater extent as it was revealed by qPCR analysis, while there was no difference in fungal DNA amount between plants inoculated with 70V-WTR and deletion mutants *70ΔAD1.4R, 70ΔAD3.4R* (Fig. 10D). Disease severity in plants infected with these AsRed transformants was calculated. At 17 dpi, plants inoculated with *Vd6ADM1R* showed symptoms of wilting, necrosis and leaf abscission with disease severity of 18.5% (Fig. 10B). In contrast, 70V-WTR, *70ΔAD1.4R*, and *70ΔAD3.4R* caused 11.5%, 4.3%, and 5.3% disease severity, respectively (Fig. 10B). Relative AUDPC values confirmed the lower disease severity observed for *70ΔAD1.4R* and *70ΔAD3.4R* (0.7% and 0.9%) and the severe virulence of *Vd6ADM1R* (6.1%) as compared to 70V-WTR (3.5%) (Fig. 10C).

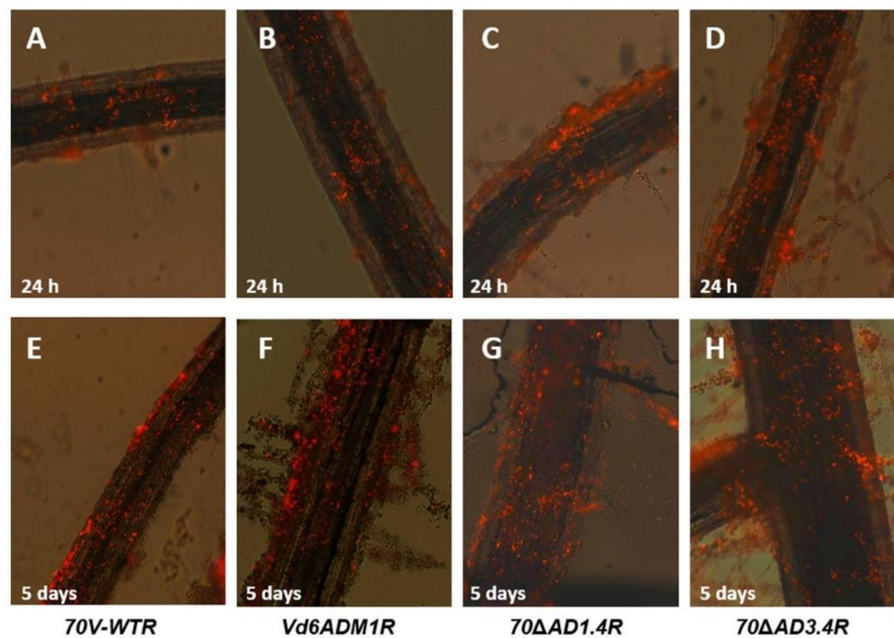


Fig. 9. Microscopic observation of tomato root colonization by 70V-WTR, *Vd6ADM1R*, *70ΔAD1.4R* and *70ΔAD3.4R*. Root surface was imaged at 1 (A-D) and 5 (E-H) days post inoculation. Magnification used for A-E was 200x, and for F, G, and H was 400x.

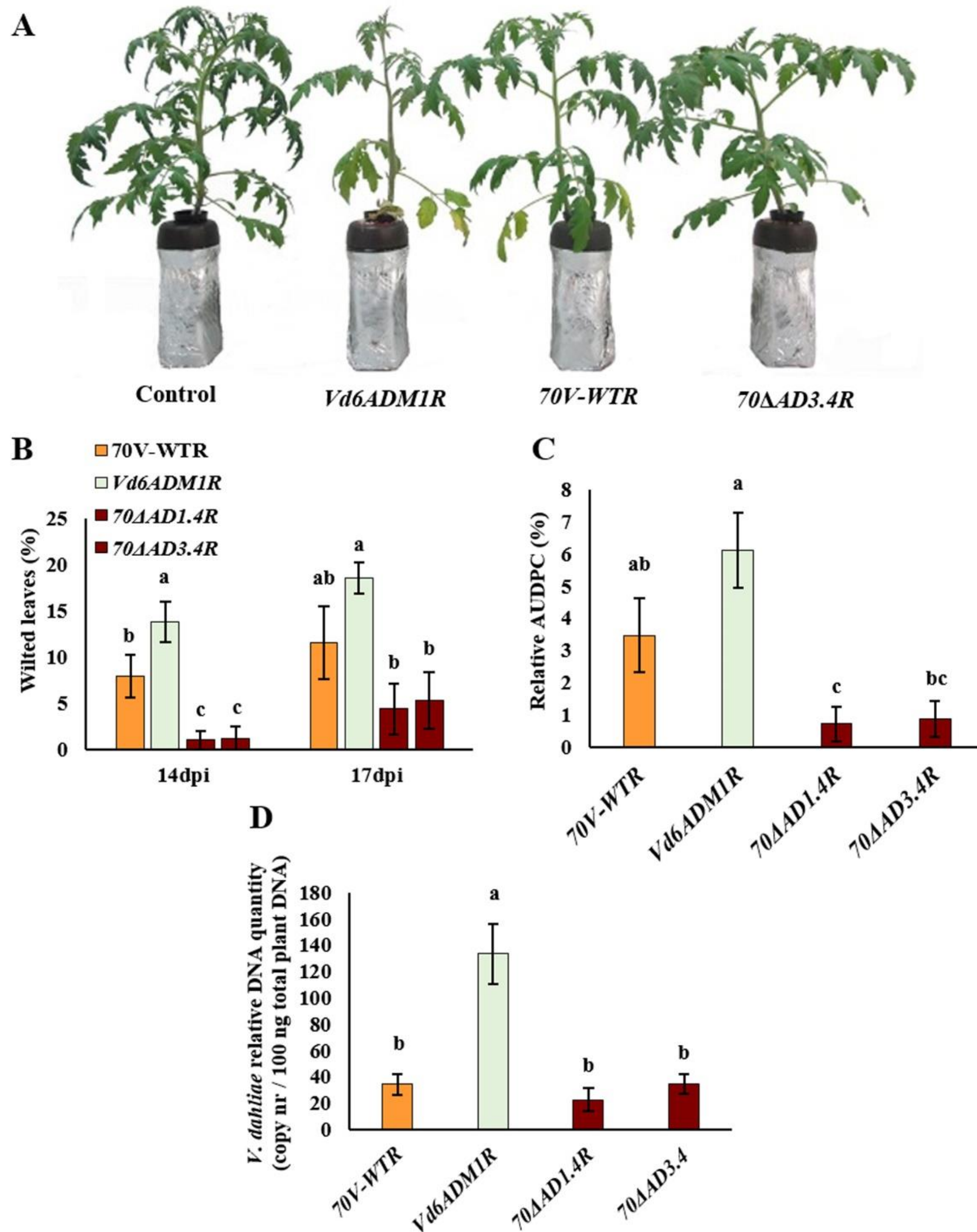


Fig. 10. Disease severity of tomato plants infected with 70V-WT and *ACCd* mutants in a hydroponic system. **(A)** Tomato plants at 17 days after inoculation with 70V-WTR, *Vd6ADM1R*, and *70ΔAD3.4R* are shown. **(B)** Disease severity at 14 and 17 days post inoculation and **(C)** the amount of disease expressed as relative AUDPC are shown. Vertical bars indicate the calculated standard errors based on six replicates. **(D)** Amount of fungal DNA in the stem of tomato plants quantified 17 days after inoculation. Vertical bars indicate the calculated standard errors of three replicates (pool of two plants per replicate per treatment). All values were subjected to analysis of variance. Different letters in **B**, **C** and **D** note statistically significant differences according to Tukey's multiple range test at $P \leq 0.05$.

***ACCd* manipulations altered fungal ACC content and affected ethylene production**

Consistent with the role of ACC deaminase in cleaving ACC, after seven days of culture, the ACC concentration in overexpression strains *Vd6ADM1* and *Vd6ADM2* was significantly lower than that in 70V-WT, and ACC levels in deletion mutants *70ΔAD1.4* and *70ΔAD3.4* were higher compared to 70V-WT (Fig 11).

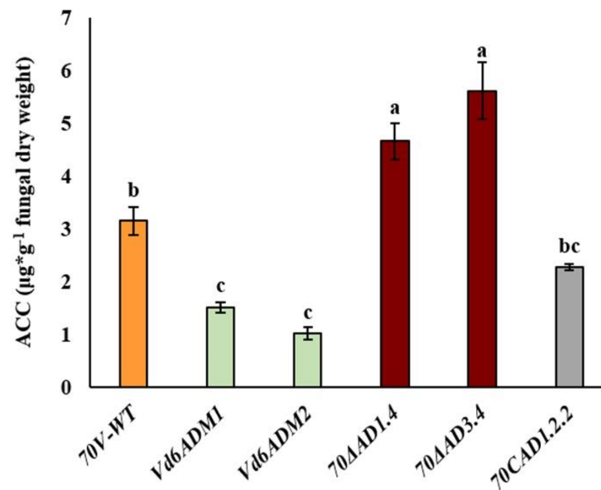


Fig. 11. Effect of *ACCd* mutations on the amount of ACC in *V. dahliae*. The amount of ACC in 70V-WT, *ACCd* deletion (*70ΔAD1.4* and *70ΔAD3.4*) and overexpression (*Vd6ADM1* and *Vd6ADM2*) mutants, and complemented deletion mutant *70CAD1.2.2* after seven days of culture are shown. Vertical bars indicate the calculated standard errors of three replicates. All values were subjected to analysis of variance. Different letters note statistically significant differences according to Tukey's multiple range test at $P \leq 0.05$.

Since ACC is the precursor of ethylene in the methionine-ACC depended biosynthetic pathway, our hypothesis was that *ACCd* deletion and overexpression would affect ethylene production. Initially, the effect of different ethylene precursors on 70V-WT ethylene production were tested. Ethylene production was stimulated only in 70V-WT cultures that had been supplemented with methionine whereas no ethylene production was detected in cultures with no supplement or when 2-oxoglutarate was added (Supplementary Fig. 7).

Subsequently, *ACCd* deletion and overexpression mutants were evaluated for their ability to produce ethylene. Ethylene production by wild type strain 70V-WT and its overexpression strains fluctuated during 14 days of cultivation (Fig. 12A). The amount of ethylene produced by *70ΔAD3.4* was higher than that of 70V-WT at multiple time

points with the maximum difference being observed at day 12. Interestingly, the amount of ethylene production by *Vd6ADM1* was also higher than that by 70V-WT at days 4, 8, and 12, but at days 6 and 14, no significant difference was observed (Fig. 12A). As methionine was shown to be the only essential precursor for ethylene production, 70V-WT and its transformants were also tested for KMBA production. All strains were found positive in KMBA production as they all formed a yellow precipitate typical of KMBA presence (Supplementary Fig. 8).

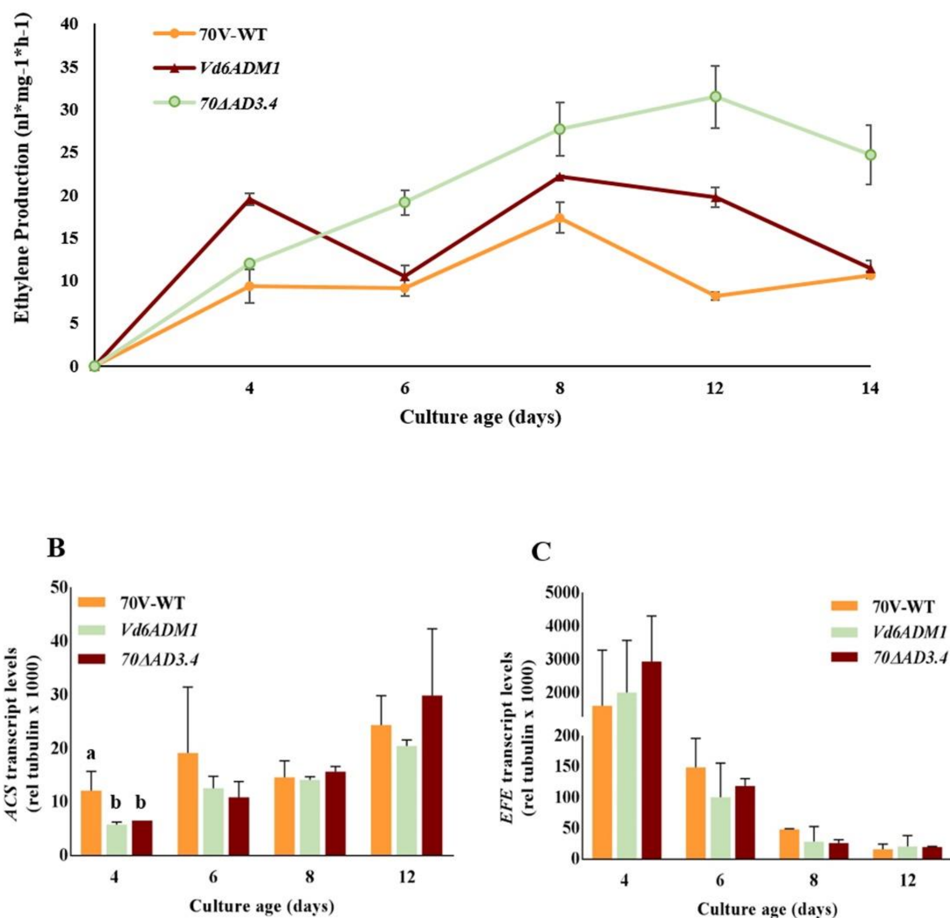


Fig. 12. Ethylene production and expression of two genes involved in ethylene biosynthesis. (A) Ethylene production over time by 70V-WT, overexpression mutant *Vd6ADM1*, and deletion mutant *70ΔAD3.4* are shown. Transcript levels of the **(B)** *ACC Synthase* and **(C)** *Ethylene Forming Enzyme* genes over time are shown. Vertical bars indicate the calculated standard errors of three replicates. All values were subjected to analysis of variance. For data that showed a significance level of $P \leq 0.05$, Tukey's post hoc analysis was also performed.

To investigate whether both mutations affect the expression of other genes potentially involved in ethylene production, transcript levels of the *ACC synthase* (*ACS*) and *Ethylene Forming Enzyme* (*EFE*) genes were determined. The amount of *ACS* transcripts in *70ΔAD3.4* and *Vd6ADM1* appeared lower than that in 70V-WT at day 4, but at days 6, 8, and 12, they looked similar (Fig. 12B). The expression of *EFE* transcripts reached its maximum at day 4 in all three strains and subsequently decreased. However, no significant differences among the strains were observed at all time points (Fig. 12C).

Discussion

Ethylene performs diverse regulatory functions in plants (Abeles et al. 1992; Johnson and Ecker 1998). Besides its role in regulating plant development and mediating the induction of plant defense responses to abiotic or biotic stresses, ethylene may act as a virulence factor for plant pathogens (Abeles et al. 1992; Van Loon et al. 2006). Effects of exogenously applied ethylene on virulence and disease development by diverse pathogens prompted studies on a putative production of ethylene by microbes in their interaction with plants (Cronshaw and Pegg 1976; Marco and Levy 1979; Tzeng and Devay 1985; Weingart et al. 2001; Cristescu et al. 2002; Chagué et al. 2006). Tzeng and DeVay (1984) reported that two *V. dahliae* isolates from cotton produced ethylene via the KMBA pathway. The non-defoliating isolate SS4 produced more ethylene than T9, a defoliating isolate, *in vitro*, but cotton plants inoculated with T9 produced more ethylene than those inoculated with SS4 (Wiese and Devay 1970; Tzeng and DeVay 1984). Since the source of ethylene in infected plants was unknown, it remains to be determined whether ethylene produced by *V. dahliae* is a virulence factor. Here, we manipulated the *ACC deaminase* (*ACCd*) gene of *V. dahliae* in two ways to investigate its role in growth, development, pathogenesis, ACC content and ethylene production. Both mutations did not significantly affect colony growth, spore production (cultured in SSN) and spore germination (Fig. 3 and Supplementary Fig. 3) but suggested the involvement of the gene and ACC in determining the severity of disease.

The ACC-dependent pathway may only play a minor role in ethylene synthesis in *V. dahliae*

In phytopathogenic fungi such as *Penicillium digitatum* and *Fusarium oxysporum*, more than one ethylene biosynthetic pathway exist (Billington et al. 1979; Fukuda et al. 1986; Hottiger and Boller 1991; Jia et al. 1999; Chagué 2010). Given the KMBA production (Supplementary Fig. 8) and the presence of genes encoding enzymes involved in the ACC and 2-OXO dependent ethylene biosynthesis pathways in *V. dahliae*, multiple pathways may participate in synthesizing ethylene. Since ACC deaminase cleaves ACC into α-ketobutyrate and ammonia (Honma and Shimomura 1978), we hypothesized that if the ACC-dependent pathway mainly produces ethylene,

overexpression of *ACCd* would lead to decreased ethylene production while its deletion would increase production. Although the amount of ethylene produced by deletion mutants was higher compared to overexpression mutants at most time points, both mutants produced more ethylene than 70V-WT (Fig. 11). This result, including the fact that all strains were found positive in KMBA production, do not support the hypothesis and also suggests that ACC, α-ketobutyrate and/or ammonia might affect the production of ethylene via other pathways. Transcript levels of the *ACC Synthase* and *Ethylene Forming Enzyme* genes in 70V-WT, overexpression mutant *Vd6ADM1*, and deletion mutant *70ΔAD3.4* were not significantly different with the exception of the *ACC Synthase* gene at one time point (Fig. 9), suggesting that the effect does not occur at the gene expression level. In a previous study it was shown that *Penicillium citrinum* uses ACC deaminase to degrade ACC to ammonia and α-ketobutyrate and that addition of ACC did not stimulate ethylene production by the fungus (Jia et al. 1999). Therefore it was assumed that ACC must have a different function in fungi than in plants.

Manipulation of *ACC deaminase* gene affects virulence of *V. dahliae*

ACC deaminase has been shown to affect plant health in different ways depending on where it is expressed. Glick et al. (1998) suggested that some ACC produced by plants is released through roots and is metabolized by root colonizing bacteria into α-ketobutyrate and ammonia, via their ACC deaminase, potentially lowering the amount of ethylene produced by plants. Several studies have shown that plants inoculated with PGPR strains expressing this enzyme form longer roots and shoots and become more resistant to a variety of abiotic stresses, including flooding, drought, heavy metals contamination, and salinity (Grichko et al. 2000; Penrose and Glick 2003a; Mayak et al. 2004; Cheng et al. 2007; Saleem et al. 2007; Glick 2014), and better protect plants from pathogenic nematodes, bacteria, fungi and oomycetes (Toklikishvili et al. 2010; Husen et al. 2011; Nascimento et al. 2013). For example, *Pseudomonas fluorescens* strain CHA0 transformed with the *ACCd* gene of *P. putida* strain UW4 displayed enhanced ability to protect cucumber from *Pythium* spp. and potato from *Pectobacterium carotovorum* subsp. *carotovorum* but failed to protect tomato from *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Wang et al. 2000). In addition engineering of plants and bacterial strains to express bacterial *ACC deaminase* genes, resulted in enhanced plant defense responses and biocontrol activity respectively (Glick 2014).

Tomato plants genetically engineered to express a bacterial ACC deaminase either in roots or upon infection resulted in significantly reduced disease severity when inoculated with a *V. dahliae* strain (Robison et al. 2001b).

The *ACCd* gene is also present in some plant pathogenic bacteria, but its role is not fully understood (Nascimento et al. 2018). The phytopathogenic bacterium *Pseudomonas brassicacearum* expressing ACC deaminase displayed growth-promoting, neutral, or phytopathogenic effect on tomato plants according to the number of bacterial cells inoculated and environmental conditions (Belimov et al. 2007). Introduction of a bacterial *ACC deaminase* gene into *Agrobacterium tumefaciens* enhanced its virulence, increasing transformation frequency of several plant species (Nonaka and Ezura 2014). Exogenous application of ethylene and ACC led to decreased ability of T-DNA transfer and low transformation rates of melon and tomato plant cells (Davis et al. 1992; Ezura et al. 1999), suggesting a role of ACC as a negative regulator of virulence. However, whether ACC deaminase or ACC affect virulence (*vir)* gene expression was not determined. Similar to *A. tumefaciens*, overexpression of the endogenous *ACCd* gene in *V. dahliae* enhanced virulence in tomato and eggplant (Figs. 5 and 7). Overexpression mutants, which contained lower levels of ACC than 70V-WT (Fig. 11), colonized the vascular system of infected plants in a greater extent than 70V-WT (Fig. 6 and 7D). In contrast, plants inoculated with deletion mutants *70ΔAD1.4* and *70ΔAD3.4*, which produced higher levels of ACC than 70V-WT (Fig. 11), showed significantly reduced symptoms than those infected with 70V-WT and overexpression mutants (Figs. 5 and 8) indicating the role of ACC as a negative regulator of virulence in Verticillium wilt disease.

Conclusion and future perspectives

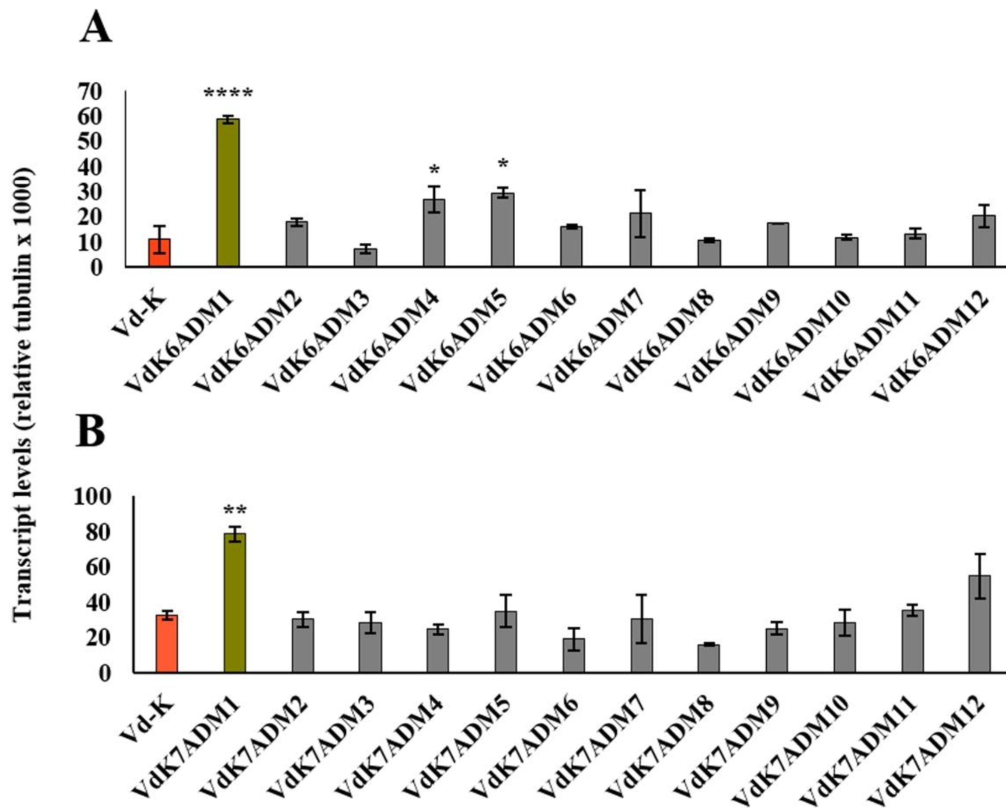
ACC deaminase activity is one of the most important mechanisms PGPR use in order to facilitate plant growth and confer protection to plants against several abiotic and biotic stresses. However, in phytopathogenic microorganisms ACC deaminase may be used to lower ACC and ethylene induced negative effects on their own gene expression or decrease ethylene regulated defense responses of the plants (Nascimento et al. 2018). The findings of the present study provide evidence for a role of *ACC deaminase* gene in pathogenicity of the soil-borne fungus *V. dahliae*. Consistent to the role of ACC deaminase in cleaving ACC, *ACCd* overexpression mutants contained

lower ACC amounts compared to 70V-WT, whereas ACC levels in *ACCd* deletion mutants were higher. Contrary to the function of ACC deaminase in PGPR, overexpression of *ACCd* gene in *V. dahliae* resulted in enhanced virulence and colonization of the vascular system of the plants, demonstrating a different role of this gene in phytopathogenic fungi. In addition *ACCd* deletion led to reduced disease development. The mode of action underlying *ACCd* role in *V. dahliae*'s virulence seems to be related to fungal ACC regulation, rather than ethylene production since both types of mutant produced more ethylene than the 70V-WT strain although they significantly differed in ACC content. However, whether fungal ACC or its degradation by *ACC* deaminase helps fungi to facilitate infection needs to be further investigated. Future transcriptomic and metabolomic profiling of both *ACCd* mutants would be essential to better explain the role of *ACCd* and ACC in the physiology and virulence of *V. dahliae*.

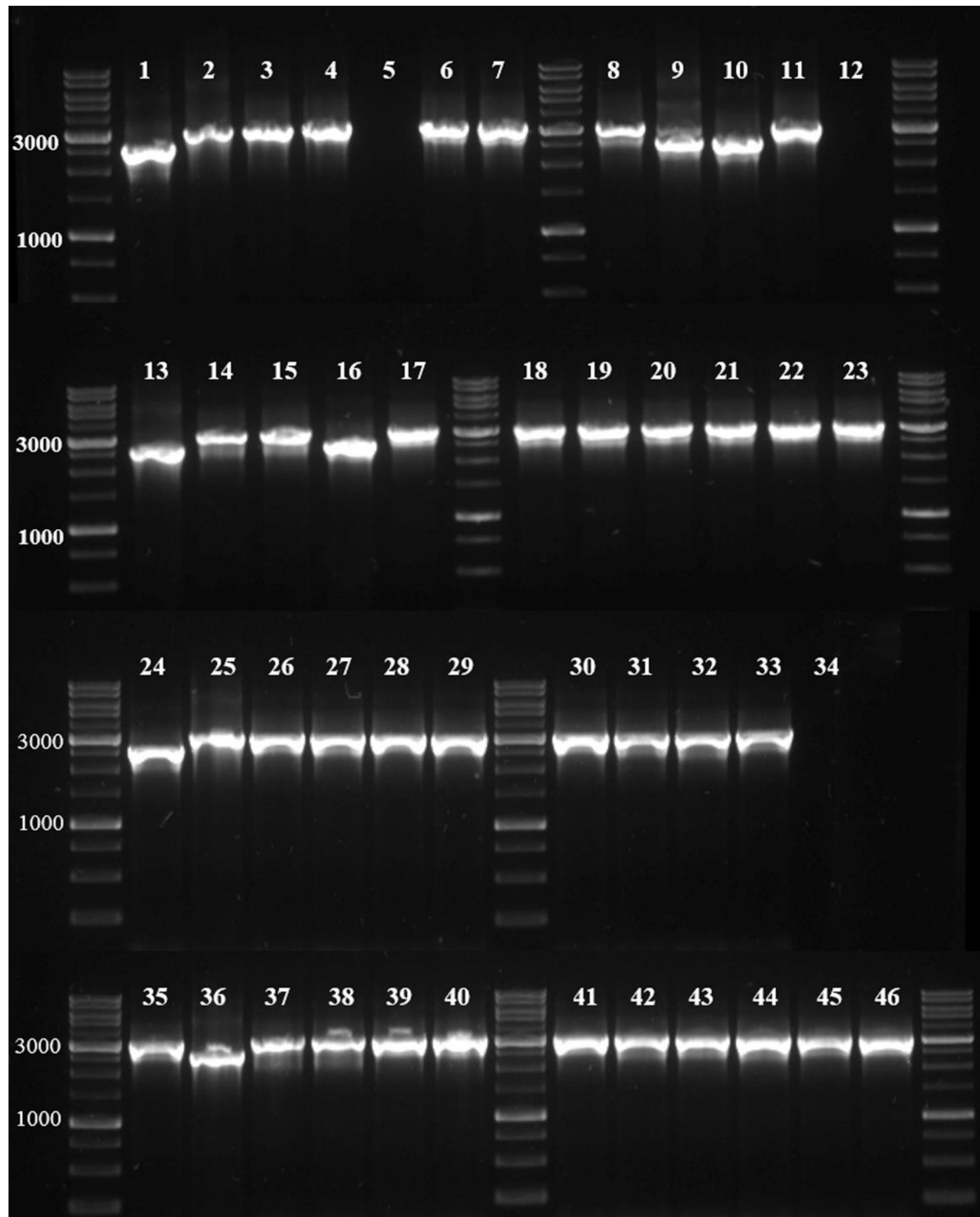
Acknowledgements

This work was supported by Dr. Dimitris Tsaltas' Startup Fund, Research Activities Fund and Researcher's Fund from the Cyprus University of Technology and by funds from the State Scholarships Foundation of Greece and the Operational Program "Education and Lifelong Learning" of the European Social Fund (ESF) within the National Strategic Reference Framework (2007-2013) with a PhD scholarship awarded to Maria-Dimitra Tsolakidou. We acknowledge Dr. Nikolaos Tzortzakis and Dr. Antonios Chrysargyris for helping hydroponic infection assays and Alexandra Triantafyllopoulou for supporting greenhouse work.

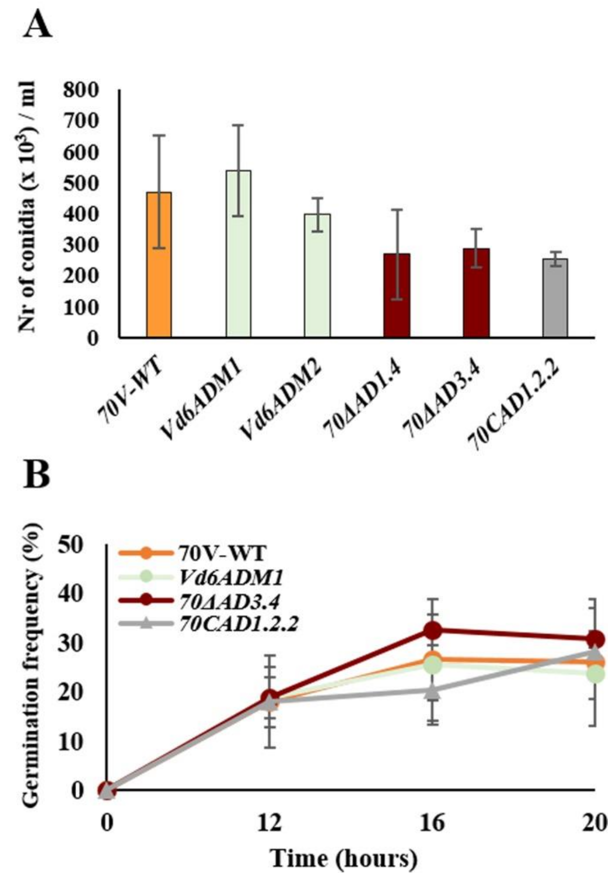
Supplementary data



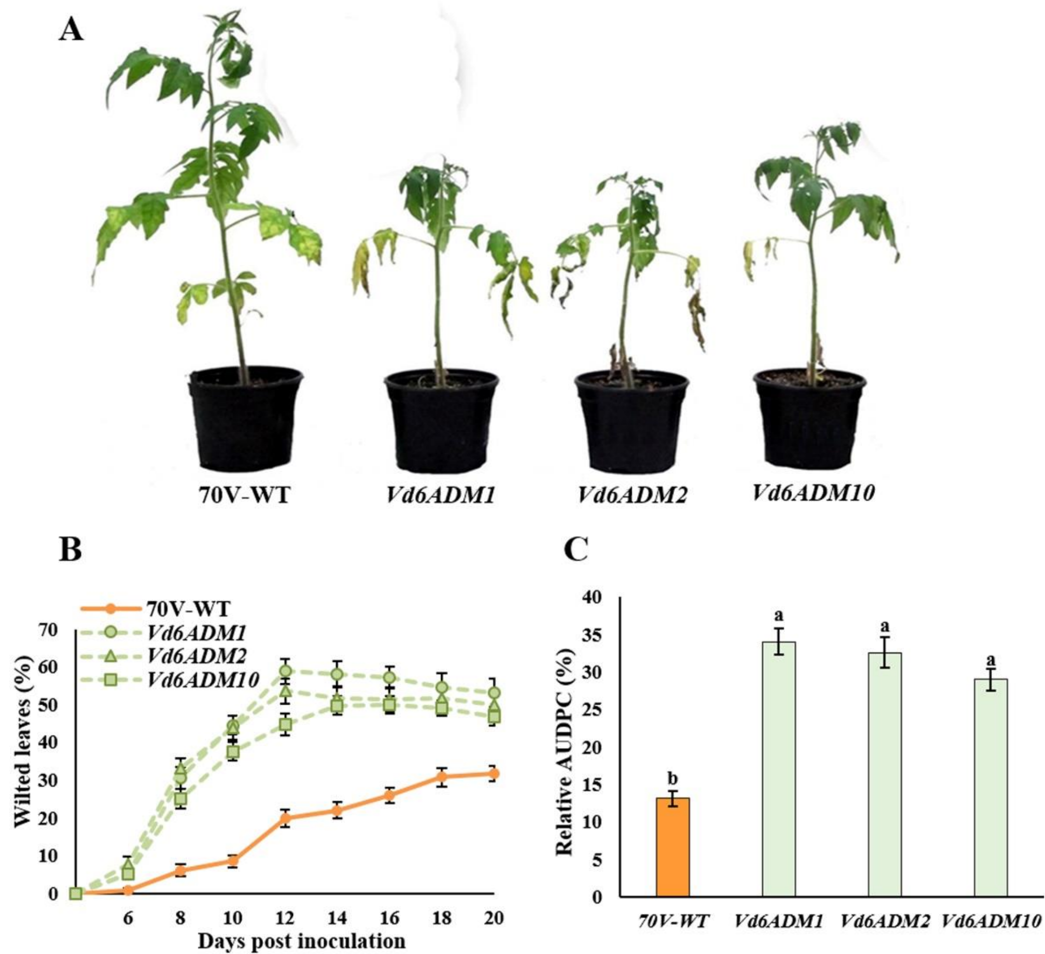
Supplementary Fig. 1. Overexpression of *ACCd* in Vd-K isolate. Transcript levels of the *ACCd* gene under **(A)** the *Magnaporthe oryzea* ribosomal protein promoter and **(B)** the *Aspergillus nidulans TrpC* promoter compared to the wild type strain Vd-K**.** Transcript levels were measured using qRT-PCR. Vertical bars indicate standard errors calculated of three replicates. Columns with asterisks indicate statistically significant differences between each mutant compared to the control Vd-K according to Dunnett's multiple comparison test (*$P \leq 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).



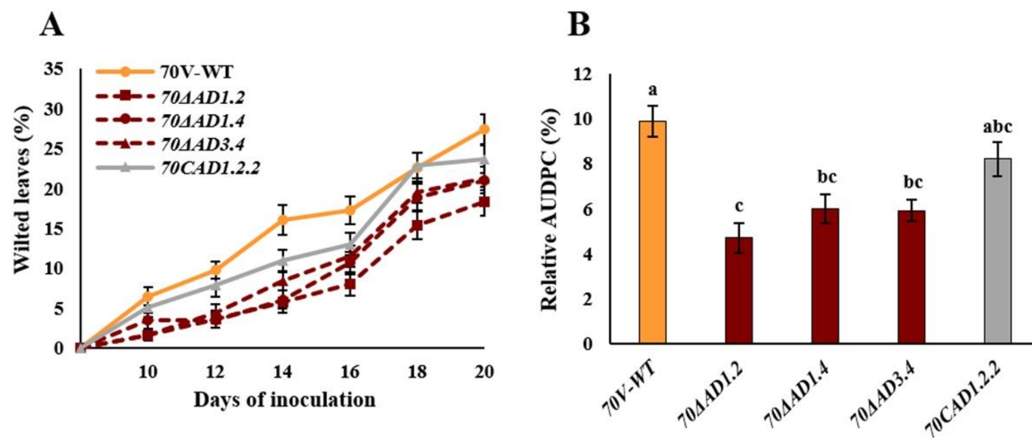
Supplementary Fig. 2. PCR confirmation of *ACCd* disruption in 38 geneticin-resistant transformants of 70V-WT. Amplification of the 2728 bp product of the *ACCd* gene with its putative promoter and terminator regions in the wild type strain 70V-WT (lanes 1, 13, 24 and 36) and in resulting ectopic transformants (lanes 9, 10, 16); Amplification of the 3171 bp fragment of the *ACCd* mutant allele in plasmid pGVdAD, used as a positive control, (lanes 2 and 25) and in resulting deletion mutants (lanes 3-11, 14-23. 26-35 and 37-46).



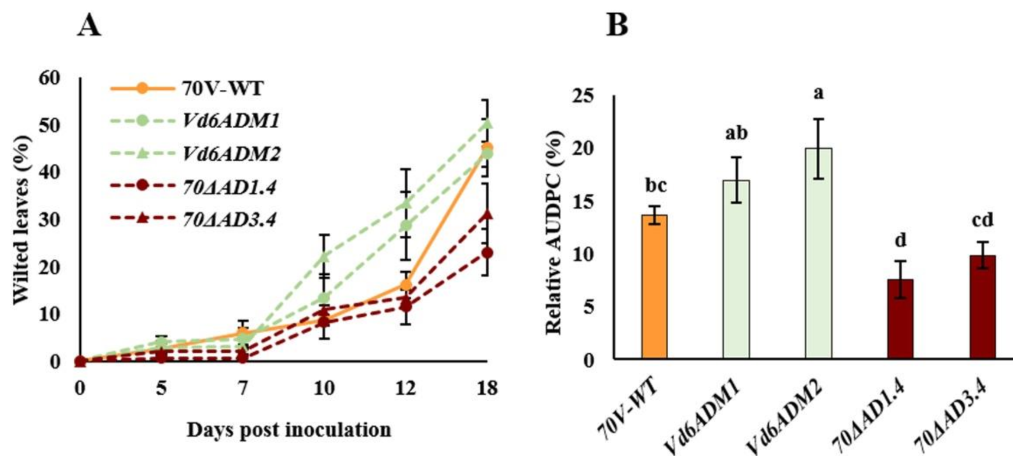
Supplementary Fig. 3. Spore production and germination in 70V-WT and *ACCd* mutants. 70V-WT, overexpression (*Vd6ADM1* and *Vd6ADM2*) and deletion (*70ΔAD1.4* and *70ΔAD3.4*) mutants, and complemented deletion mutant *70CAD1.2.2* were cultured in liquid SSN medium to measure their spore production. **(A)** Spore production and **(B)** the percentage of germinated conidia over time for each strain are shown. Vertical bars indicate the calculated standard errors of three replicates. All values were subjected to analysis of variance, and no statistically significant differences were found.



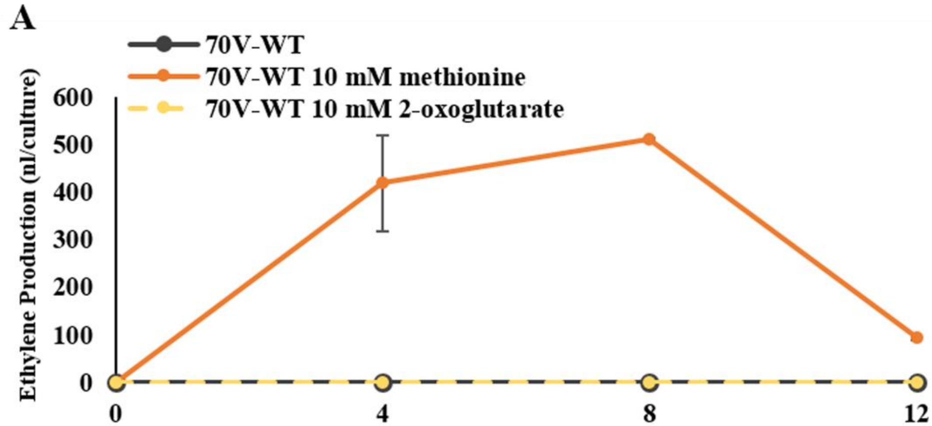
Supplementary Fig. 4. Disease symptoms in tomato plants infected by 70V-WT and *ACCd* overexpression mutants in growth room. Tomato plants were infected with 70V-WT and three overexpression mutants. (**A**) Symptoms at 14 days after infection, (**B**) disease progression over time and (**C**) relative AUDPC are shown. Vertical bars indicate the standard errors based on twenty replicates. All values were subjected to analysis of variance. Different letters in **C** note statistically significant differences according to Tukey's multiple range test at P≤ 0.05.



Supplementary Fig. 5. Disease symptoms in tomato plants infected by 70V-WT and its *ACCd* deletion mutants in growth room. Eggplants were infected with 70V-WT, two deletion mutants (*70ΔAD1.4* and *70ΔAD3.4*) and a complemented deletion mutant (*70CAD1.2.2*). **(A)** Disease progression over time and **(C)** relative AUDPC are shown. Vertical bars indicate the standard errors based on twenty replicates. All values were subjected to analysis of variance. Different letters in **B** note statistically significant differences according to Tukey's multiple range test at P≤ 0.05.



Supplementary Fig. 6. Disease severity of tomato plants infected by 70V-WT and its *ACCd* mutants in greenhouse. (A) Disease progression over time and **(B)** amount of disease expressed as relative AUDPC are shown. Vertical bars indicate the calculated standard errors of ten replicates. All values were subjected to analysis of variance. Different letters in B note statistically significant differences according to Tukey's multiple range test at P≤ 0.05.



Supplementary Fig. 7. Effect of different precursors on ethylene production. Ethylene production over time by 70V-WT in liquid Czapek's medium, or in medium supplemented with 10 mM methionine or 2-oxoglutarate. Vertical bars indicate the calculated standard errors of three replicates.

Supplementary Fig. 8. Production of KMBA by 70V-WT and *ACCd* mutants in medium supplemented with methionine. **(A)** 70V-WT, overexpression mutants (*Vd6ADM1* and *Vd6ADM2*), and **(B)** deletion mutants (*70ΔAD1.4* and *70ΔAD3.4*) produced KMBA, as evidenced by the formation of yellow precipitate. No such precipitate was formed in non-inoculated medium (labeled as Meth), while yellow precipitate was formed in medium containing 10 mM KMBA (labeled as Meth + KMBA).

Supplementary Table 1. Primers used in this study.

Name	Sequence[1]	Target[2]	Reference
ACD_EX-F1	5'-ATGAATTCATTCAGGCGGTATCCAGGTCAACAACA-3'	*ACC deaminase* (VDAG_10392)	This study
ACD_P-F1	5'- ATCTGCAGAGGCGGTATCCAGGTCAACAACA-3'		This study
ACD_K-R1	5'-AAGGTACCGGGGGTCATCCATGCCTATCACT-3'		This study
ACD_X-F2	5'-AATCTAGCGGAGGGCGACATCACA-3'		This study
ACD_H-R2	5'-TTTAAGCTTCATCCTCCACCTGGGCTCTTTC-3'		This study
ACD_X-C-F1	5'-ATTCTAGAAGGCGGTATCCAGGTCAACAACA-3'		This study
ACD_OX_F	5'-CCACGATCTAGACCACACCACCACCACATC-3'		This study
ACD_OS_R	5'-CCATTTGCATGCATTGACCCTCGCCCTCTC-3'		This study
ACD_Rt1_F	5'-GCCAAACGCGAGGACTGC-3'		This study
ACD_Rt1_R	5'-CACGCCGCCAATGCTGAC-3'		This study
ACS_Rt1_F	5'-AACTCGGGCCGCCACAACATT-3'	*ACC synthase* (VDAG_05021.1)	This study
ACS_Rt1_R	5'-ACAGGGCGCGGAGCAAGGAT-3'		This study
EFE_RT1_F	5'-ACGACGTCGGTGCCCTGTTTGTTC-3'	*Ethylene / Succinate forming enzyme* (VDAG_09492.1)	This study
EFE_RT1_R	5'-TGCGCTGGGGGTAGTTCCTGATGA-3'		This study
ITS1-F	5'-AAAGTTTTAATGGTTCGCTAAGA-3'	*ITS1*	Gardes and Bruns, (1993)
ST-VE1-R	5'-CTTGGTCATTTAGAGGAAGTAA-3'	18S rDNA	Lievens et al. (2006)
VdBt-F	5'-TTCCCCCGTCTCCACTTCTTCATG-3'	*β-tubulin* (VDAG_10074.1)	Tzima et al. (2012)
VdBt-R	5'-GACAAGATCGTTCATGTTGGACTC-3'		
LeTUB-F	5'-GATTTGCCCACTAACCTCTCGT-3'	*Lycopersicon esculentum β-tubulin gene* (DQ205342)	Pantelides et al. (2009)
LeTUB-R	5'-ACCTCCTTTGTGCTCATCTTACCC-3'		

[1] Underlined sequences correspond to restriction enzyme sites introduced to facilitate subsequent cloning.

[2] With the exception of LeTUB-F and LeTUB-R, all primers correspond to *V. dahliae* genes.

Chapter 3

Investigation of the role of ACC as potential regulator of plant defense against the soil-borne pathogen *Verticillium dahliae*

Parts of this chapter have been published to 'Molecular Plant-Microbe Interactions' (DOI: 10.1094/MPMI-07-18-0203-R).

Abstract

The plant hormone ethylene plays a vital role in coordinating plant developmental processes and stress resistance. Plants can regulate their hormonal equilibrium through their own regulatory mechanisms, however their hormonal balance can be also affected by several plant-associated microbes. Certain microorganisms can reduce plant ethylene levels by regulating the pool of 1-amino-cyclopropane-1-carboxylic acid (ACC), the immediate ethylene precursor, via ACC deaminase (ACCd) enzyme. Recently, several studies have reported ACC to act as signaling molecule controlling plant growth and defense responses. In this chapter we investigated the role of ACC as a positive regulator of defense against the soil-borne fungus *Verticillium dahliae*. Application of *V. dahliae ACCd* mutants to tomato plants showed that *ACCd* overexpressing and deletion mutants can alter ACC levels in the roots of plants. To investigate whether ACC accumulation in the root system of the plants can affect the development of Verticillium wilt disease, wild type and ethylene insensitive tomato and *Arabidopsis* plants were root-treated with ACC prior to infection with *V. dahliae.* It was found that both tomato and *Arabidopsis* ACC-treated plants displayed less severe symptoms than untreated controls. Moreover, ACC application on the roots of Col-0 and *etr1-1* plants i*n vitro* was found to trigger root hair formation and the expression of hormone-dependent defense genes. In conclusion, our results suggest that ACC acts as defense regulator against Verticillium wilt disease, however future transcriptional studies are required to shed more light on the mechanism underpinning ACC triggered defense responses.

Introduction

In nature, plants are threatened by numerous abiotic and biotic stresses (Boyd et al. 2013; Carroll et al. 2018; Zandalinas et al. 2018). Since plants are sessile organisms, they have developed sophisticated defense mechanisms to overcome a wide range of pathogens (including fungi, bacteria, viruses, oomycetes and nematodes) herbivore insects and adverse environmental conditions, essential for their survival. Plant defense mechanisms consist of an array of structural barriers and multifaceted molecular and biochemical changes such as production of antimicrobial metabolites, programmed hypersensitive cell death, transcriptional reprogramming and expression of defense-related genes (Van Loon et al. 2006; Pieterse et al. 2009). In addition, plants have evolved an innate immune system, represented as the 'zigzag' model that helps them to recognize their intruders and defend themselves against pathogen attack or microbial invasion (Jones and Dangl 2006; Wu et al. 2014).

Plants produce a wide variety of hormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA) that play key roles in the induction of defense mechanisms since they act as signaling molecules regulating plant responses to herbivore insects, pathogens and beneficial microorganisms (Bari and Jones 2009; Erb and Glauser 2010; Pieterse et al. 2012). The hormonal signal that is stimulated depends on the type of the attacker. For instance JA is a key regulator of plant defenses to necrotrophic pathogens, chewing insects and wound responses, while SA is mainly induced in response to biotrophic pathogens and phloem-sucking insects. In the model plant *Arabidopsis thaliana*, the JA pathway consists of two distinct and antagonistic branches, the MYC- and the ERF-branch, which are co-regulated by ABA and ET, respectively. The ET-regulated ERF-branch of the JA pathway is associated with plant defenses against necrotrophic pathogens, while the ABA-regulated MYC-branch is associated with wounding and herbivore insects attack (Anderson 2004; Verhage 2011; Kazan and Manners 2013). These hormone-mediated signaling pathways can communicate both in a synergistic or antagonistic manner providing plants the tools to fine-tune their immune response (Pieterse et al. 2009; Robert-Seilaniantz et al. 2011).

Ethylene is a principal modulator in many physiological processes of plants ranging from seed germination to fruit ripening and also mediates plant responses to external stimuli (Ecker and Davis 1987; Abeles et al. 1992; Johnson and Ecker 1998). In

addition, treatments of plants with either ethylene and/or its precursors or inhibitors have not only demonstrated its implication in plant defense but also in symptom expression (Pegg 1976; Robison et al. 2001a; Van Wees et al. 1999; Thomma et al. 1999). However, the effect of this hormone in a particular plant-pathogen interaction depends on the lifestyle and the infection strategies of the pathogen, the nature of the physiological reactions that are triggered by the pathogen and the lifestyle of the host (Thomma et al. 1999). In plants, ethylene is synthesized from methionine through two intermediates, S-adenosyl-methionine (S-AdoMet) and 1-amino-cyclopropane-1-carboxylic acid (ACC) (Adams and Yang 1979) with ACC being considered as the rate limiting step of ethylene biosynthesis in plants (Yang and Hoffman 1984). Recently, studies have converged on a model suggesting that ACC could be more than just the precursor of ethylene and act as a signaling molecule, regulating plant growth and development (Xu et al. 2008; Tsuchisaka et al. 2009; Tsang et al. 2011; Yoon and Kieber 2013; Van de Poel and Van Der Straeten 2014; Vanderstraeten and Van Der Straeten 2017). According to Vanderstraeten and Van Der Straeten (2017) one of the ethylene-independent functions of ACC is its secretion from plant roots into the rhizosphere to attract and interact with PGPR. Some of these PGPR produce an enzyme called ACC deaminase, which cleaves ACC into α-ketobutyrate and ammonia, and can promote plant growth presumably by lowering plant ethylene levels (Honma and Shimomura 1978; Glick 2014).

Previously it was shown that mutant strains of the soil-borne fungus *Verticillium dahliae* overexpressing the endogenous *ACC deaminase* gene (*ACCd*) had lower ACC contents and exhibited enhanced virulence in tomato and eggplant. On the contrary *ACCd* deletion mutants of *V. dahliae* produced higher levels of ACC and caused significantly less severe symptoms compared to the wild type strain, indicating the role of fungal ACC as a virulence regulator (**Chapter 2**). In this study we initially investigated whether *ACCd* mutants of *V. dahliae* can alter plant ACC levels after infection and if accumulation of ACC in the roots of the plants could result in enhanced resistance against *V. dahliae*. For this purpose *Arabidopsis* and tomato plants were root-treated with ACC prior to infection with *V. dahliae* and disease development was evaluated. We also examined whether ACC could be involved in disease development in an ethylene independent way by evaluating how ethylene insensitive *Arabidopsis* and tomato plants pre-treated with ACC respond to infection by *V. dahliae*. Expression

analysis of defense-related genes in the roots of ACC-treated wild type and ethylene insensitive *Arabidopsis* plants was also assessed *in vitro* in an effort to shed light on the low disease severity of the ACC-treated plants. Our results indicate that ACC acts as defense regulator against *Verticillium* wilt disease, however whether it functions in a non-ethylene dependent manner needs to be further elucidated.

Materials and Methods

Fungal isolates and culture conditions

70V-WT, a *Verticillium dahliae* isolate from tomato (Pantelides et al. 2010b) and its ACC deaminase (*ACCd*) transformants and Vd-R, a *V. dahliae* isolate from radish (Tjamos et al. 2005), were used throughout this study. For long term storage, these isolates were preserved as a conidial suspension (4 x 10^7 conidia ml^{-1}) in 25 % glycerol at -80°C. They were activated by culturing on PDA (Potato Dextrose Agar, OXOID) for 5 days at 25°C. *ACCd* overexpression strains and deletion mutants were cultured on PDA supplemented with 50 µg ml^{-1} geneticin (Life Technologies) or hygromycin B (Duchefa Biochemies) to maintain pure culture of fungal cells expressing the plasmid constructs used for transformation.

For infection assays, fungal isolates were cultured in liquid Sucrose Sodium Nitrate (SSN) medium (Sinha and Wood 1968) for 5 days at 25°C by shaking at 140 rpm using an orbital incubator. Before plant inoculation, cultures were passed through cheesecloth to remove mycelia, and the conidial concentration was adjusted to 1x10^7 conidia ml^{-1}.

ACC measurement in plant tissues

ACC was determined in roots and shoots of tomato plants: (a) inoculated with 70V-WT and *ACCd* transformants as described in "Conditions for plant growth and infection" paragraph and (b) root-drenched with 10 ml of 100 µM ACC. Plants were uprooted 2 and 4 days after infection and twelve hours after ACC treatment respectively, rinsed thoroughly with sterile, deionized water and lyophilized for 5 days. All samples were immediately frozen by immersing them in liquid nitrogen before freeze-drying. The amount of ACC was measured using the method described in Lizada and Yang (1979) in which the amount of ACC formed was measured via its conversion to ethylene. Ethylene was determined by gas chromatography. The amount of ethylene in 1 ml air sample was measured using 7890A Agilent gas chromatographer (Agilent Technologies), equipped with a flame ionization detector and an Agilent stainless steel column (2mm id x 2m) packed with Porapak Q 80/100 mesh, as described by Petrou et al. (2013) with minor modifications. Helium was used as carrier gas under a constant inlet pressure of 20 psi. Inlet and oven temperatures were maintained at 80°C, and detector temperature was set at 200°C. The total runtime was 3 min with ethylene being

detected at 1.8 min. Calibration was performed using 1 ppm ethylene. For plant ACC determination three biological samples (pool of two plants per treatment) were used.

Conditions for plant growth and infection

Seeds of tomato mutant line *Never ripe* (*Nr*; Tomato Genetics Resource Center, University of California, Davis) and cv. Ailsa Craig (*Solanum lycopersicum* Mill. cv. Ailsa Craig, Thompson and Morgan Ltd) and seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0, Syngenta, Basle, Switzerland) and mutant line *etr1-1* (Nottingham Arabidopsis Stock Centre, Nottingham, UK) were stored at 4°C. For tomato infection assays, seedlings were initially grown in a plastic tray and were then transferred into 10.5 cm-diameter pots (Teku, VCG 10.5 pots) with each pot containing approximately 450 ml soil (Plantaflor Potting Soil, Germany). The pots were placed in a controlled-environment growth room set at 65–70% RH, 25°C, and a 16-h photoperiod with a photon flux density of 200 ± 20 μmol m^{-2} s^{-1}. Plants were watered every two days to maintain 70% of soil humidity. For *Arabidopsis* pathogenicity assays, seeds were sown directly into 9 cm-diameter pots (Teku, VCH 9 pots), containing approximately 330 ml soil (Plantaflor Potting Soil, Germany) per pot. After 4 days stratification at 4°C, the pots were placed in a growth room set at 22ºC, 65–70% RH, and a 16-h photoperiod with photon flux density of 100 ± 20 μmol m^{-2} s^{-1}. Plants were watered every two days to maintain 70% of soil humidity.

ACC involvement in plant defense against *V. dahliae* was examined using WT and *Nr* tomato plants and Col-0 and *etr1-1 Arabidopsis* plants. For these experiments six plants per genotype per treatment were used. A solution of 100 µM ACC was applied by root drenching (10 ml per plant) to two-week-old plants 12 hours prior to fungal inoculation. The pathogen was inoculated by root drenching with 10 ml conidial suspension ($1x10^7$ conidia ml^{-1}). Control plants were inoculated with 10 mL sterile distilled water. The experiments were repeated twice (a total of twelve plants per treatment).

Disease severity was calculated by the number of wilted leaves as a percentage of the total number of leaves for each plant. Symptoms were periodically recorded for up to 20 (tomato) and 26 (*Arabidopsis*) days post inoculation (dpi) and plotted over time to generate the area under the disease progress curve (AUDPC) using the trapezoidal integration method (Campbell and Madden 1990). Disease was expressed as a

percentage of the maximum possible area for the whole period of the experiment, referred to as relative AUDPC.

In vitro plant growth assays

Arabidopsis thaliana accession Col-0 and the mutant line *etr1-1* were used for the *in vitro* plant growth assays. Seeds were surface sterilized (Van Wees et al. 2013) and sown in square plates (120 x 120 x 17 mm) containing 1 x MS medium supplemented with 0.5% sucrose. After 4 d of stratification at 4°C, plates were transferred in growth chamber (22°C; 8h light, 16h dark; light intensity 100 μmol m^{-2} s^{-1}) and positioned vertically. To determine the effect of ACC on plant morphological parameters, 9 days-old plants were inoculated with 10 μl of 100 μM ACC just below the root tip and were further grown for another 10 days. Roots of control plants were inoculated with 10μl of ultra-pure sterile H_2O. For shoot fresh weight measurements, seedlings were sectioned at the root-shoot junction, and the weight of three groups of 8 excised shoots per treatment was measured on an analytical balance. For root length measurements, digital images of petri dishes of *Arabidopsis* seedlings were captured and the primary root length of 3 groups of 8 seedlings per treatment was calculated with ImageJ software (https://imagej.nih.gov/ij/). For root hair length measurements 5 individual roots were obtained for each treatment and in each root the length of 56–95 root hairs was estimated by ImageJ. In total, approximately 370 root hairs were measured per treatment. Root hair density was determined as the average root hair number in a 1 mm^2 root segment. Root hair length and density were estimated using photographs obtained from the primary root segment located 1.0 cm above the root tip using a binocular microscope (Olympus SZX16) with an attached digital camera (ColorView II, Olympus Soft Imaging System GmbH).

In vitro experiments for gene expression analysis

A. thaliana Col-0 and *etr1-1* seeds were surface sterilized, sown, stratified and grown as described before. To examine the effect of ACC in the induction of plant defense responses, roots of 16 days-old Col-0 and *etr1-1* plants were covered with 20 μl of 100 μM ACC and collected for gene expression experiments 6 hours after ACC treatment. The experiment included 3 biological replicates (8 plants each) per treatment. Roots of control plants were covered with 20μl of ultra-pure sterile H_2O. Total RNA

was extracted from roots ground in the presence of liquid nitrogen as previously reported (Oñate-Sánchez and Vicente-Carbajosa 2008). RNA samples were treated with DNase (TAKARA) to eliminate DNA contamination and first-strand cDNA was synthesized using PrimeScript RT reagent kit (TAKARA).

Expression levels of defense related genes was assessed by real-time quantitative PCR (qPCR) reactions in an IQ5 thermocycler (Bio-Rad) using the KAPA SYBR FAST qPCR kit (KAPA Biosystems). PCR cycling consisted of an initial step of 95°C denaturation for 3 min, followed by 40 cycles at 95°C for 3 s and 60°C for 30 s to calculate cycle threshold (Ct) values. The primer specificity and the formation of primer-dimers were monitored by dissociation curve analysis. The *A. thaliana Protein Phosphatase 2A Subunit A3* (At1g13320) gene was used as an internal standard to normalize small differences in cDNA template amounts. For data analysis, average threshold cycle (Ct) values were calculated for each gene of interest (Pfaffl 2001) on the basis of three independent biological samples. Primers used in gene expression analysis are listed in Supplementary Table 1.

Statistical analysis

Statistical analyses were performed using R package (R 2016, https://www.r-project.org, version 3.3.1). Data obtained were subjected to analysis of variance (ANOVA) and significant differences between individual means were then determined using Sidak's test or Tukey's post hoc pairwise comparison test at the 5% confidence level. Student's *t* test was used for analyzing gene expression data.

Results

***ACC deaminase* mutants of *V. dahliae* affect ACC content of tomato plants**

The less virulent *ACCd* deletion mutants of *V. dahliae* were shown to have a higher ACC content compared to the wild type strain 70V-WT while *ACCd* overexpression strains produced significantly lower amounts of ACC compared to both 70V-WT and *ACCd* deletion mutants (Chapter 2, Fig. 11). To investigate if *ACCd* manipulation in *V. dahliae* could not only alter ACC levels of the pathogen but also affect the ACC levels of tomato plants upon infection, the ACC content was determined in the roots and shoots of infected plants during the early stages of infection. At 2 and 4 dpi, ACC content in roots of plants infected with *Vd6ADM1* and *Vd6ADM2* overexpression strains was significantly less compared to plants infected with 70V-WT and the mock-inoculated controls (Fig. 1A). At 2 dpi ACC content in roots of plants infected with *70ΔAD1.4* and *70ΔAD3.4* deletion mutants was not significantly different compared to 70V-WT and mock-inoculated plants but was significantly higher than that of the overexpression strains (Fig 1A). However, at 4 dpi, root ACC levels of 70V-WT and mock-inoculated controls were reduced, while ACC content in roots of *70ΔAD1.4* and *70ΔAD3.4* infected plants remained at the same levels, showing a significantly higher ACC content compared to the wild type and mock-inoculated plants (Fig. 1A). ACC amount was also assayed in the shoots of infected plants 2 and 4 days after inoculation and no significant difference was observed among all treatments (Fig. 1B), indicating that the effect caused by *ACCd* transformants is localized at the infection site.

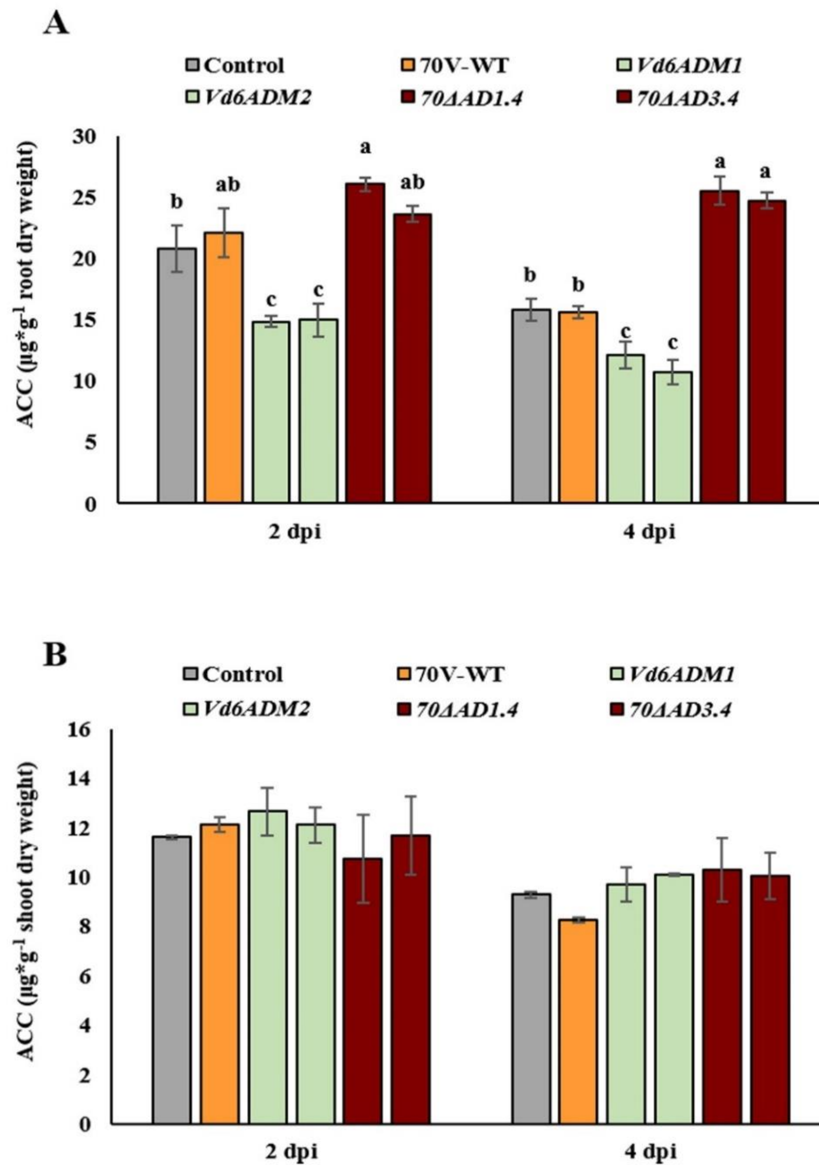


Fig. 1. ACC content in tomato plants infected with *V. dahliae* and its *ACCd* mutants. ACC content in **(A)** roots and **(B)** shoots of mock-inoculated tomato plants and plants infected with 70V-WT, *Vd6ADM1* and *Vd6ADM2* overexpression strains and *70ΔAD1.4* and *70ΔAD3.4* deletion mutants are shown. Vertical bars indicate the calculated standard errors of three replicates (pool of two plants per replicate per treatment). All values were subjected to analysis of variance. For data that showed a significance level of $P \leq 0.05$, Tukey's post hoc analysis was also performed.

Exogenous application of ACC prior to infection cause less disease symptoms in tomato and *Arabidopsis* plants

Since *ACCd* deletion mutants not only produced higher ACC amounts than that of 70V-WT wild type strain but also increased the ACC levels in the roots of tomato plants, we hypothesized that accumulation or reduction of ACC in the root system of the plants might affect their response against *V. dahliae*. To determine whether ACC can affect plant defense against *V. dahliae*, roots of WT and *Nr*, a mutant line insensitive to ethylene, tomato plants were root drenched with ACC prior to infection with isolate 70V-WT. After ACC treatment, both plant genotypes showed less severe symptoms and slower disease development compared to untreated plants (Fig. 2A). At 16 dpi, disease severity of the untreated plants was about 23%, while in ACC treated WT and *Nr* plants was only 10% and 8%, respectively. (Fig. 2B). The relative AUDPC, that was adopted to comprehensively evaluate the disease severity, confirmed the previous observations. Relative AUDPC values for ACC treated WT and *Nr* plants were 5.3 and 6.5% respectively compared to the non ACC treated WT and *Nr* controls showing AUDPC values of 8.7 and 9.7% respectively (Fig. 2C).

To further investigate whether ACC affects *V. dahliae* disease development, *Arabidopsis* was used as an additional host. Roots of *A. thaliana* ecotype Col-0 and the ethylene insensitive mutant *etr1-1*, were also root treated with ACC prior to infection with the *V. dahliae* isolate Vd-R. Overall, both ACC-treated Col-0 and *etr1-1* plants showed less severe symptoms and slower disease development than mock-inoculated controls (Fig. 3A). The first disease symptoms appeared in the form of wilting in Col-0 at 12 dpi. At 18 dpi, ~26% of leaves in Col-0 and *etr1-1* plants displayed wilt symptoms, while ACC treated Col-0 and *etr1-1* plants displayed only 10% and 12% disease severity, respectively (Fig. 3B). The relative AUDPC analysis revealed that ACC treated Col-0 and *etr1-1* plants exhibited statistically less disease symptom progress (6 and 8% respectively) compared to the control plants (11%), indicating an increased disease resistance of ACC-treated plants against *V. dahliae* (Fig. 3C).

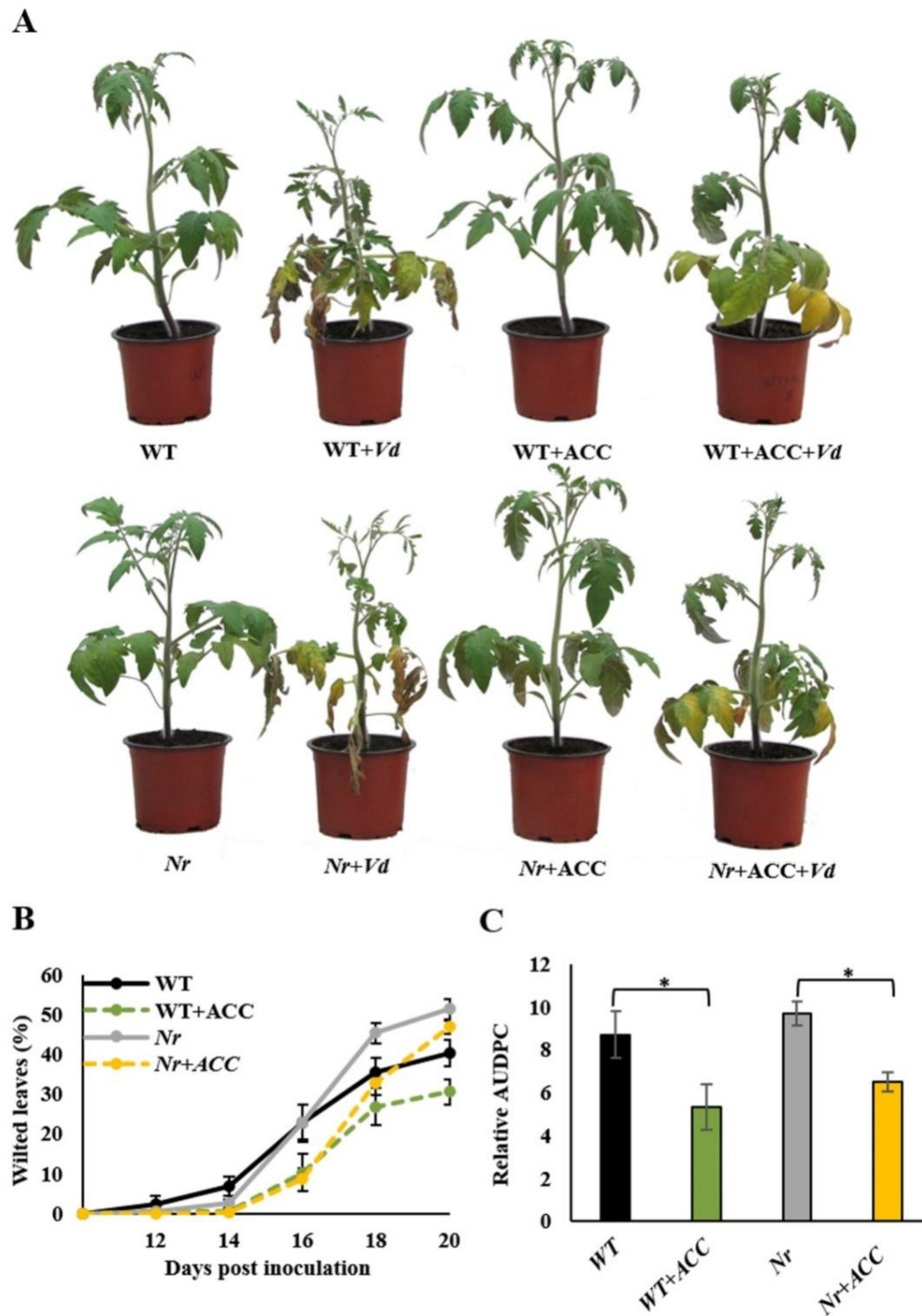


Fig. 2. Effect of exogenous application of ACC on disease development in tomato plants infected with 70V-WT. Plants were treated with 100 μM ACC or water by root drenching (10 ml per plant) 12 hours prior to fungal inoculation. **(A)** Symptoms at 18 days after inoculation, **(B)** disease progress curves, and **(C)** the amount of disease expressed as relative AUDPC are shown. Vertical bars indicate the calculated standard error of twelve replicates. All values were subjected to analysis of variance. Asterisks in C note statistically significant differences according to Sidak's test (*$P \leq 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

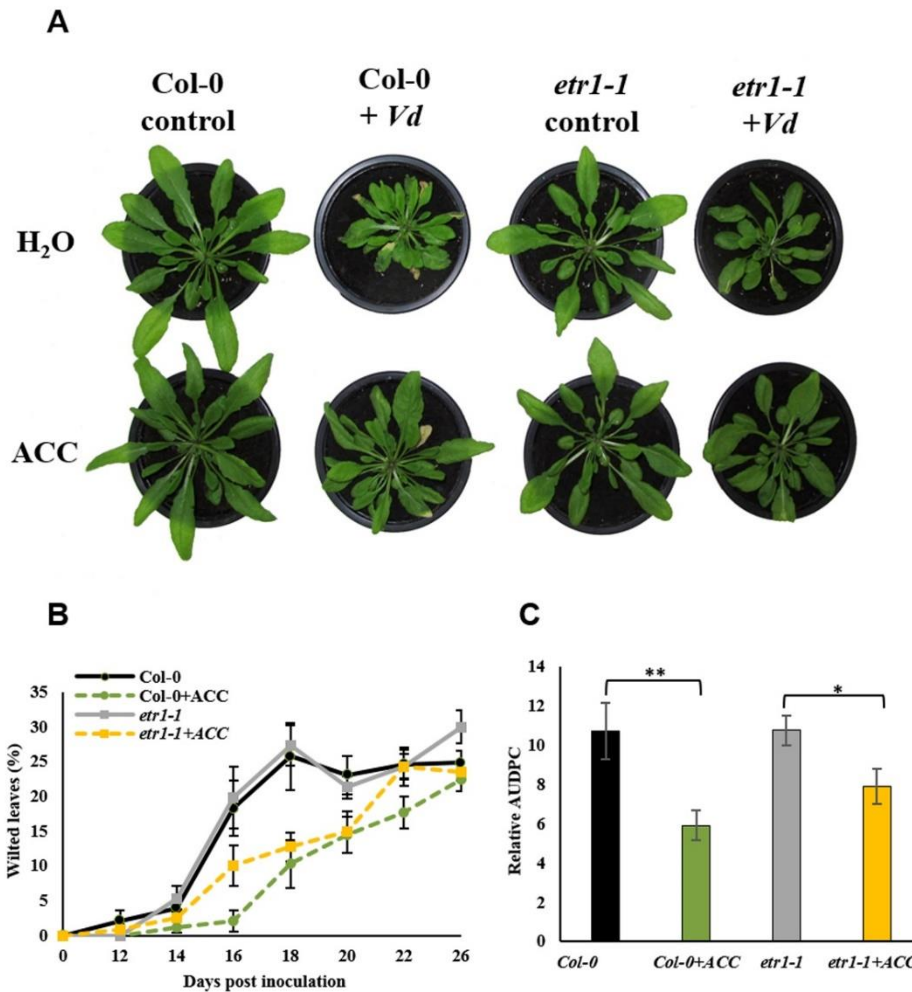


Fig. 3. Effect of exogenous application of ACC on disease development in *Arabidopsis* plants infected with Vd-R. Plants were treated with 100 μM ACC or water by root drenching (10 ml per plant) 12 hours prior to fungal inoculation. **(A)** Symptoms at 18 days after inoculation, **(B)** disease progress curves, and **(C)** the amount of disease expressed as relative AUDPC are shown. Vertical bars indicate the calculated standard errors of twelve replicates. All values were subjected to analysis of variance. Asterisks in C note statistically significant differences according to Sidak's test (*$P \leq 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

Exogenous application of ACC affects certain root growth parameters of Col-0 and *etr1-1* seedlings without inhibiting shoot growth

ACC is known to inhibit primary root growth through ethylene production (Ruzicka et al. 2007). For this reason we examined whether growth characteristics of Col-0 plants and plants of the ethylene insensitive line *etr1-1* are affected after root treatment with ACC. Plant growth parameters, such as shoot biomass, primary root length, number of lateral roots, root hair length and density were determined in 19 days-old seedlings, 10

days after application of 100 μM ACC. ACC treatment led to primary root length inhibition of Col-0 seedlings (Figs 4A and 4C) but no effect was observed in the formation of lateral roots (Fig. 4B) or in shoot fresh weight of ACC treated Col-0 seedlings compared to the non-ACC treated controls (Supplementary Figs 1A and 1C).

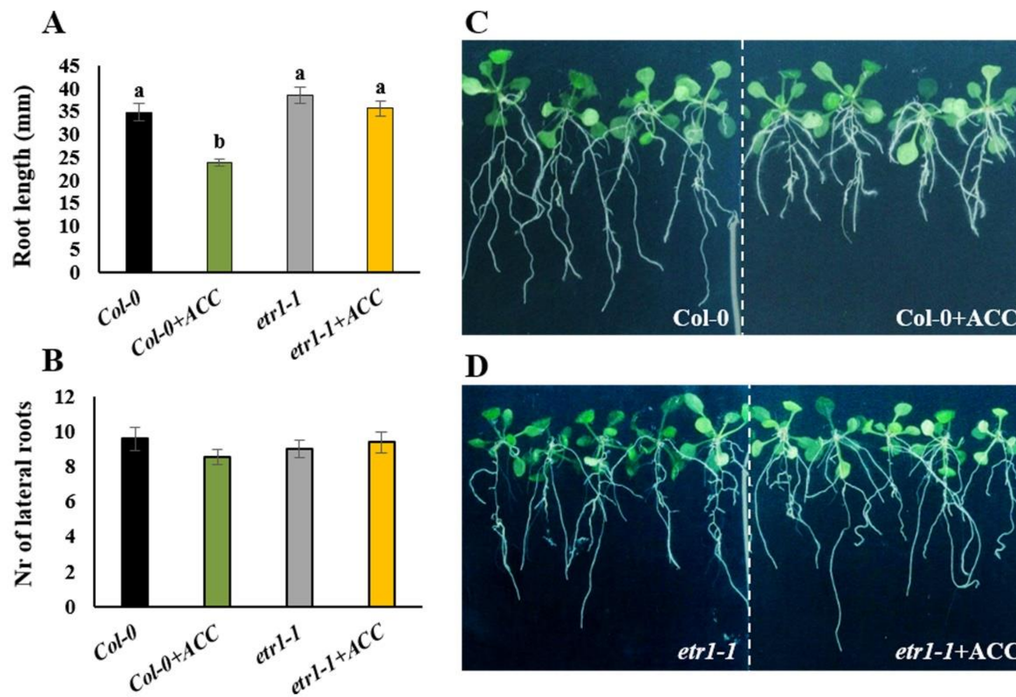


Fig. 4. Effect of ACC on root system architecture of *Arabidopsis* seedlings. (A) Primary root length, **(B)** number of lateral roots and representative images of **(C)** Col-0 and **(D)** *etr1-1* seedlings root system 10 days after 100 μM ACC or water treatment are shown. Vertical bars in **A** and **B** indicate the calculated standard error of 24 replicates. All values were subjected to analysis of variance. Different letters in **A** and **B** note statistically significant differences according to Tukey's multiple range test at $P \leq 0.05$.

On the contrary, root hair length of Col-0 plants was stimulated by ACC compared to the non-ACC treated plants (Figs 5C-F). The mean length of root hairs in ACC-treated plants was significantly longer compared to the non ACC-treated controls (Fig. 5A), however the density of mature root hairs of plants treated with ACC was not altered (Fig. 5B).

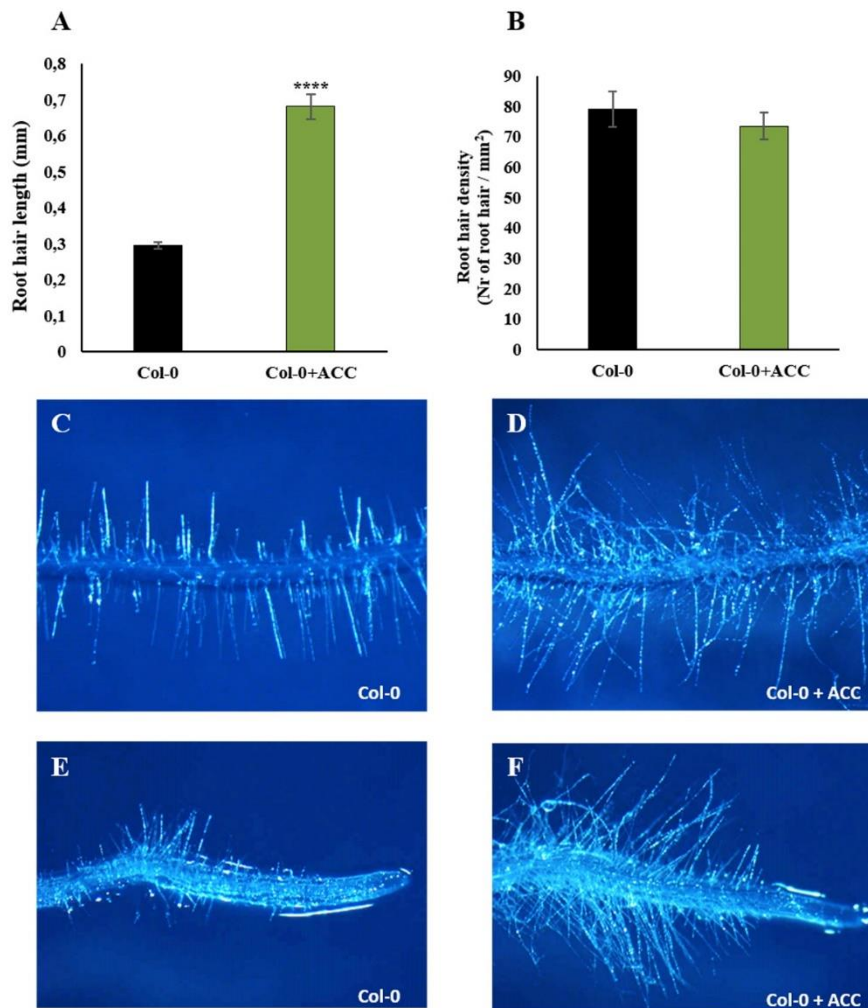


Fig. 5. Effect of ACC on Col-0 root hair formation. (A) Average root hair length, **(B)** root hair density and representative images of root hair formation in root segments located 1 cm above the root tip **(C)** and **(D)** and in root tips **(E)** and **(F)** of Col-0 seedlings 10 days after 100 µM ACC or water treatment are shown. Vertical bars in **A** and **B** indicate the calculated standard error of 5 replicates. Asterisks indicate statistically significant differences according to Student's *t* test (*****P* < 0.0001).

In the ethylene insensitive *etr1-1* plants, ACC had no impact on the primary root length (Figs 4A and 4D), the number of lateral roots (Fig. 4B), the fresh weight of the shoots (Supplementary Figs. 1B and 1C) and in root hair density (Fig. 6B). Interestingly, root hair length was also significantly increased in ACC-treated *etr1-1* seedlings (Figs 6A and 6C-F), indicating that ACC might affect root hair growth, independently of ethylene perception by plants.

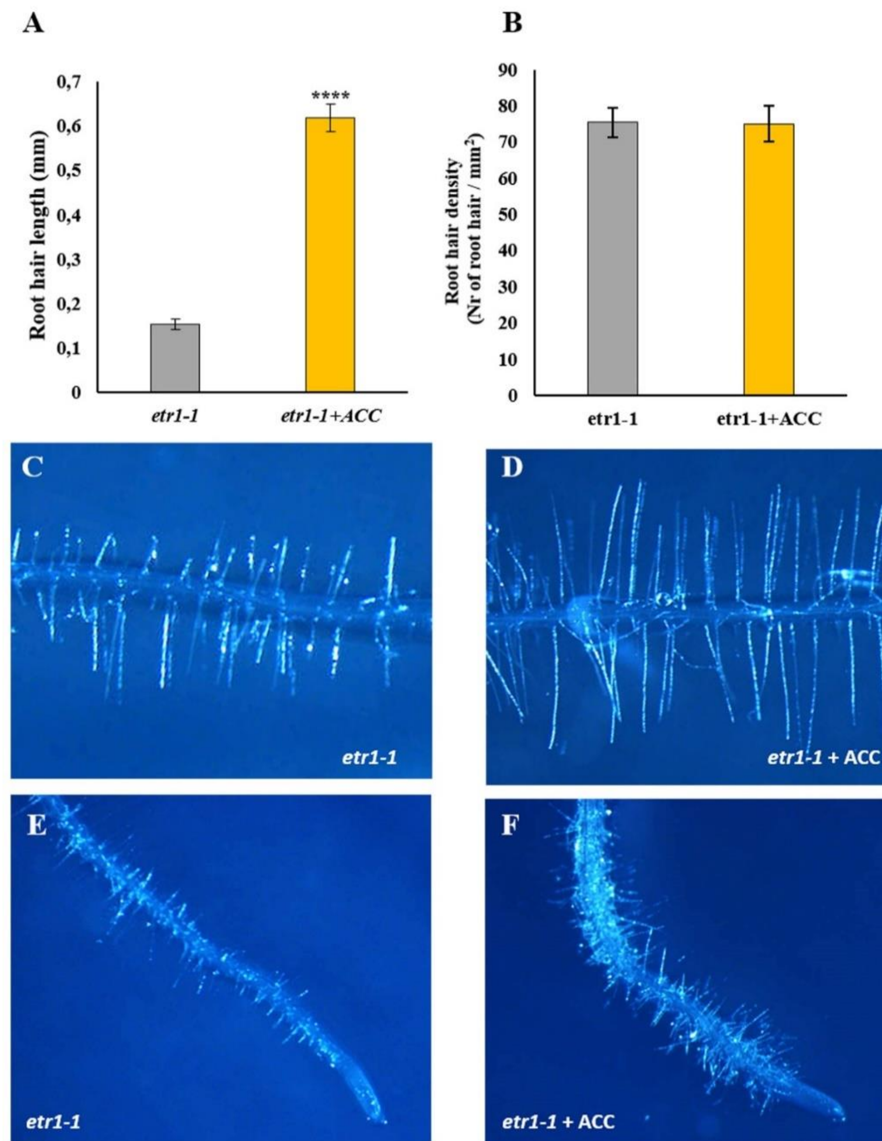


Fig. 6. Effect of ACC on *etr1-1* root hair formation. **(A)** Average root hair length and **(B)** root hair density and representative images of root hair formation in root segments located 1 cm above the root tip **(C)** and **(D)** and in root tips **(E)** and **(F)** of *etr1-1* seedlings 10 days after 100 μM ACC or water treatment are shown. Vertical bars in **A** and **B** indicate the calculated standard errors of 5 replicates. Asterisks indicate statistically significant differences according to Student's *t* test (****$P < 0.0001$).

Exogenous application of ACC in the roots of *Arabidopsis* triggers SA, JA and ET depended defense responses

In an attempt to interpret the phenotype observed in ACC pathogenicity assays and to investigate whether application of ACC to the roots can trigger plant defense mechanisms, gene expression analysis of several hormone-related marker genes was performed in roots of Col-0 and *etr1-1* plants pretreated with 100 μM ACC.

In ACC-treated roots of Col-0 plants, expression of *PR3*, *PR4* and *ERF1* genes, which are markers for the ET-modulated branch of the JA defense pathway, was significantly up-regulated compared to the untreated controls (Figs 7C, 7D and 7F). Expression levels of the *VSP2* gene of the ABA-regulated branch of JA pathway and expression levels of the SA-depended *PR2* and *PR5* genes were significantly down-regulated, highlighting the antagonistic interaction among the JA-ABA, SA and JA-ET pathways (Figs 7B, 7E and 7H). Interestingly, ACC application induced the transcription of *PR1* gene which is an indicator of activation of defense responses regulated by the SA pathway (Fig. 7A). Application of ACC had no effect in root expression of *RD22* and *MYC2* genes, which are involved in ABA response, and *MYB72*, a root specific transcription factor of Induced Systemic Resistance(ISR) (Figs 7G, 7I and 7K). The expression of the transcription factor *MYB51*, which is responsible for indole glucosinolates synthesis, was significantly higher than that of the controls (Fig 7J).

Expression levels of defense genes were also examined in ACC-treated roots of *etr1-1* mutants to investigate whether ACC can induce defense mechanisms independently of ethylene (Fig. 8). Although ACC-treated *etr1-1* plants showed enhanced resistance against *V. dahliae* in pathogenicity assays, gene expression analysis in ACC-treated roots of *etr1-1* plants showed that 6 hours after ACC application only *ERF1* gene was up-regulated, while *VSP2* and *MYB72* genes were down regulated compared to the untreated controls (Figs 8F, 8H and 8K). No significant difference was observed in the rest of the genes tested.

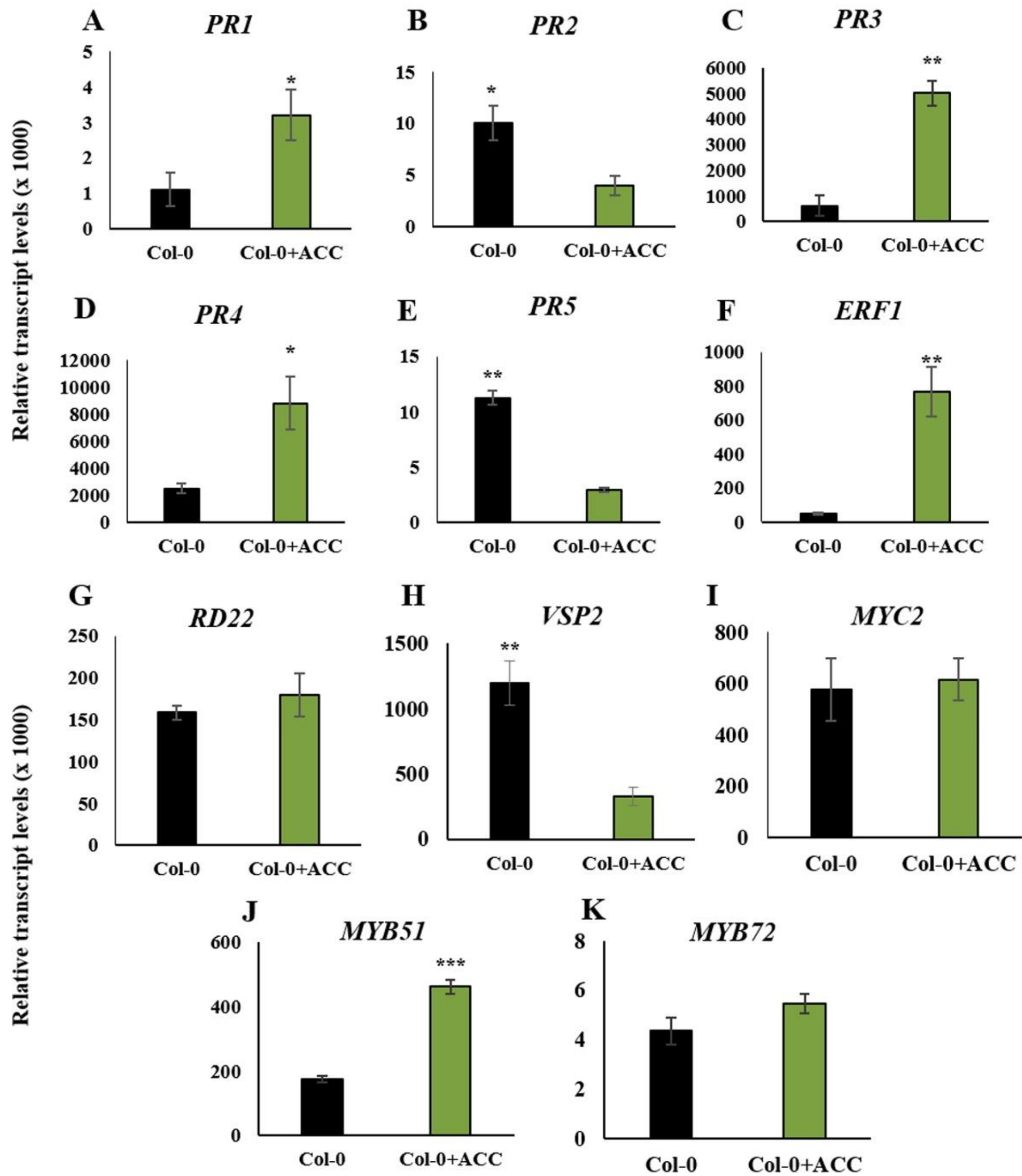


Fig. 7. Transcription of hormone-related marker genes in roots of Col-0 plants after ACC treatment. Expression levels of defense marker genes were measured 6 h after 100 μM ACC application in the roots of Col-0 plants. Data were normalized over the *Protein Phosphatase 2A Subunit A3* housekeeping gene (At1g13320). Vertical bars indicate the calculated standard error of three biological replicates (pool of eight roots per replicate per treatment). Asterisks indicate statistically significant differences according to Student's *t* test (*$P \leq 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

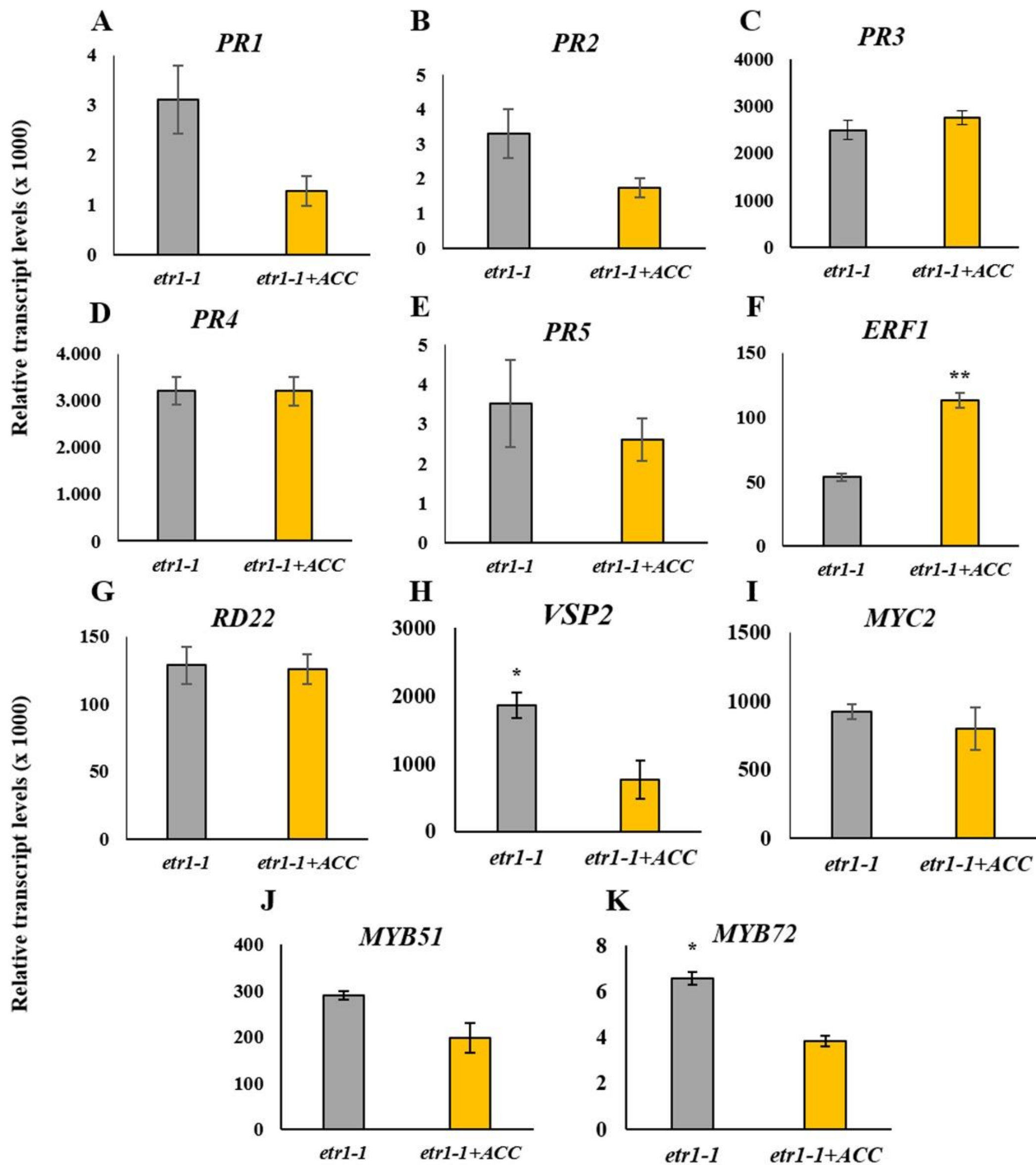


Fig. 8. Transcription of hormone-related marker genes in roots of *etr1-1* plants after ACC treatment. Expression levels of defense marker genes were measured 6 h after 100 µM ACC application in the roots of *etr1-1* plants. Data were normalized over the *Protein Phosphatase 2A Subunit A3* housekeeping gene (At1g13320). Vertical bars indicate the calculated standard error of three biological replicates (pool of eight roots per replicate per treatment). Asterisks indicate statistically significant differences according to Student's *t* test (*$P \leq 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

Discussion

Plant hormones such as jasmonic acid (JA), salicylic acid (SA), ethylene (ET) and abscisic acid (ABA) mediate signal transduction pathways that play a pivotal role in the activation and regulation of plant immune responses (Bari and Jones 2009; Robert-Seilaniantz et al. 2011; Pieterse et al. 2012; De Vleesschauwer et al. 2014; Papadopoulou et al. 2018). Plants regulate their hormonal equilibrium through their own regulatory mechanisms, however their hormonal balance can be also affected by several plant-associated microbes (Ravanbakhsh et al. 2018).

Several beneficial and pathogenic microorganisms are able to increase plant ethylene levels either by directly synthesizing ethylene or by inducing enzymes, like ACC synthase, which participate in ethylene biosynthesis (Suganuma et al. 1995; Weingart and Völksch 1997; Ravanbakhsh et al. 2018). Microorganisms can also decrease plant ethylene levels. Certain PGPR can promote plant growth and confer resistance to plants against several abiotic and biotic stresses, by reducing plant ethylene levels via their ACC deaminase activity. ACC deaminase degrades ACC, the immediate precursor of ethylene, resulting in lower plant ethylene concentrations (Glick et al. 1998; Glick 2014).

ACC acts as a potential regulator of plant defense against *V. dahliae*

A number of studies have demonstrated the beneficial effect of ACC deaminase-producing PGPR against several plant pathogens (Toklikishvili et al. 2010; Husen et al. 2011; Nascimento et al. 2013). However it seems that the role of this gene may differ depending on where it is expressed. Previously, it was shown that inoculation of tomato and eggplant with *ACC deaminase* (*ACCd*) mutants of the soil-borne fungus *V. dahliae*, containing low amounts of ACC, enhanced disease severity while deletion strains, containing higher levels of ACC than the wild type strain, showed significantly reduced symptoms (Chapter 2). These findings suggested that ACC accumulation in the roots of plants during *V. dahliae*-host interaction may affect the outcome of the disease. To test this hypothesis, in this study, it was primarily assessed whether these *ACCd* transformants could alter plant ACC levels after infection of plants. In other studies investigating the beneficial effects of ACC deaminase-producing PGPR in plant growth, it was shown that these bacteria can lower plant ACC levels and thus ethylene

production (Madhaiyan et al. 2007; Yim et al. 2013, 2014). Consistent with these studies and with the role of ACC deaminase in cleaving ACC, in the present study it was shown that roots of tomato plants infected with *ACCd* overexpression strains displayed less ACC amounts compared with the mock-inoculated controls (Fig. 1A). In contrast, ACC levels in roots of plants inoculated with *ACCd* deletion mutants were higher compared to the controls (Fig. 1A). In addition, the amount of ACC in tomato plants pre-treated with ACC was significantly higher in both roots and shoots, twelve hours after inoculation compared to mock-inoculated controls (Supplementary Fig. 2).

The increased ACC levels in the roots of plants infected with *ACCd* deletion mutants and in roots of ACC treated plants raised the question of whether exogenous application of ACC to the roots could mimic the phenotype observed in infection by *ACCd* deletion mutants and confer resistance to plants against *V. dahliae*. In a previous study, Robison et al. (2001a) suggested that long-term inhibition of ethylene production through AVG (aminoethoxyvinylglycine) treatment, an ACC inhibitor, combined with a transient burst of ethylene at the time of infection upon ACC application, could reduce Verticillium wilt disease symptoms in tomato plants. However in the same study, no significant differences were observed in disease symptoms when *V. dahliae* infected tomato plants were sprayed only with ACC. Our results suggest that ACC acts as a potential positive regulator of defense, since its root application significantly reduced disease in WT and *Nr* tomato plants (Fig. 2) and in Col-0 and *etr1-1 Arabidopsis* plants (Fig. 3). Foliar application of ACC to *Arabidopsis thaliana* plants has been also shown to enhance resistance against *Pseudomonas syringae* pv. *tomato* (Pieterse 1998; Van Wees et al. 1999; Pieterse et al. 2000), also supporting the role of ACC as a positive regulator of defense.

An ACC regulated mechanism which could explain the phenotype observed in pathogenicity assays, is the inhibition of root cell elongation. In this process ACC induces responses such as the production of reactive oxygen species (ROS), hydroxyproline-rich glycoproteins (HPRGs), cell-wall strengthening components and callose deposition contributing to cell elongation arrest but also decreasing cell wall loosening agents making cells stronger hence less susceptible to pathogen colonization (Staal et al. 2011; Markakis et al. 2012). Interestingly, most of these ACC-regulated mechanisms have been also observed in immune responses induced by microbial or pathogen associated molecular patterns (MAMPs or PAMPs) (Boller and Felix 2009),

suggesting an ACC-regulated mechanism controlling simultaneously plant development and defense.

ACC may function as a signal molecule in plant defense

Several studies suggested that ACC could be more than just the precursor of ethylene and act as a signaling molecule regulating plant development and defense responses (Xu et al. 2008; Tsuchisaka et al. 2009; Tsang et al. 2011; Yoon and Kieber 2013; Van de Poel and Van Der Straeten 2014). For example, root cell elongation in *A. thaliana* appears to be controlled by ACC, in a non-ethylene dependent way (Xu et al. 2008; Tsang et al. 2011). ACC also plays a possible role in phytohormone crosstalk (Van de Poel and Van Der Straeten 2014; Nascimento et al. 2018). In this study it was shown that exogenous application of ACC to the roots of Col-0 and ethylene insensitive *etr1-1* plants *in vitro*, caused enhanced mean root hair length in both plant lines (Figs. 5 and 6). This result is consistent with the literature since it is well established that auxins, ethylene and ACC are positive regulators of root hair development (Tanimoto et al. 1995; Pitts et al. 1998). However the fact that ACC pre-treated *etr1-1* mutants also exhibited enhanced mean root hair length compared to the mock-inoculated controls, might support an ethylene-independent function for ACC.

ACC application on the roots of Col-0 and *etr1-1* plants *in vitro* was also found to trigger hormone-dependent defense responses which might explain the enhanced resistance shown by ACC-treated tomato and Arabidopsis plants against *V. dahliae* in pathogenicity assays. Being the precursor of ethylene in plants, ACC is correlated with triggering ethylene mediated defense responses. Van Wees et al (1999) showed that when Arabidopsis leaves were dipped in ACC, the marker genes *PR3*, *PR4* and *PDF1.2*, for the ET-modulated branch of the JA pathway, were prominently induced while *PR1*, *PR2* and *PR5* which are SA-depended were slightly activated. They also suggested that ACC concentrations above 1 mM were less effective in inducing resistance, probably due to enhanced ET-induced senescence. Here, in ACC pre-treated Col-0 plants, ET-dependent genes *PR3*, *PR4* and *ERF1* were significantly up-regulated in the roots compared to the untreated controls, while genes of the ABA-modulated branch of the JA pathway were down-regulated (Fig. 7). Surprisingly and despite the antagonistic relationship of ET and SA, *PR1* was up-regulated in the roots of ACC-treated Col-0 plants (Fig. 7), suggesting either that *PR1* induction does not necessarily require SA

accumulation or that ACC could trigger plant defense mechanisms which are ET independent. *PR1* up-regulation was also observed in *Brassica rapa* roots after ethephon application indicating that roots may respond differently in specific hormonal pathways (Papadopoulou et al. 2018). In the same study the authors also suggested that *PR1* induction was probably due to ET elicitation, however they could not eliminate the possibility that ethephon could have an ethylene independent effect. In our study, ACC application on the roots of *etr1-1* plants, resulted in the overexpression of the *ERF1* gene only despite the enhanced resistance shown by ACC treated *etr1-1* plants against *V. dahliae*. Since the *etr1-1* mutant line is ethylene insensitive and *ERF1* gene is induced either by JA or/and ET, a possible explanation could be that its up-regulation occurred via activation of JA defense mechanisms without ET involvement.

Due to the complexity of hormonal crosstalk and given that even subtle changes in the production of plant hormones can significantly affect plant-microbe interactions, it needs to be further investigated how ACC production and regulation in both the fungal and plant sides can influence the outcome of infection.

Conclusion and future perspectives

This study supports the notion that ACC can function as positive regulator of plant defense. *ACCd* deletion mutants of *V. dahliae* increased ACC content in the roots of infected plants while *ACCd* overexpression strains decreased ACC levels, suggesting that ACC levels in the roots may be regulated by the pathogen which could lead to differential disease development. Exogenous application of ACC decreased symptoms in both tomato and *Arabidopsis* plants challenged with *V. dahliae*. Moreover, Verticillium wilt disease was also reduced in ACC pre-treated ethylene-insensitive *Nr* and *etr1-1* plants, implying defence-related effects of ACC to be ethylene-independent. Additionally, interesting results were obtained from *in vitro Arabidopsis* experiments. ACC application enhanced mean root hair length in Col-0 and *etr1-1* plants and also induced the expression of SA, JA and ET-depended defense genes. It was also shown that ACC may act in an ethylene-independent way as its application in ethylene insensitive plants negatively affected *V. dahliae* disease development and also ACC application in the roots of *in vitro* grown *etr1-1* plants led to the overexpression of *ERF1* gene. Future experiments should focus on unravelling the molecular and biochemical processes underlying ACC accumulation in the roots during plant-pathogen

interactions. It would be therefore essential to initially detect the signaling pathway underlying ACC triggered defense mechanisms by performing pathogenicity assays using mutant lines of *Arabidopsis* in different hormonal pathways. Transcriptional profiling of ACC pre-treated plants during infection would also provide valuable information on the mechanisms involved in the defense responses of plants.

Acknowledgements

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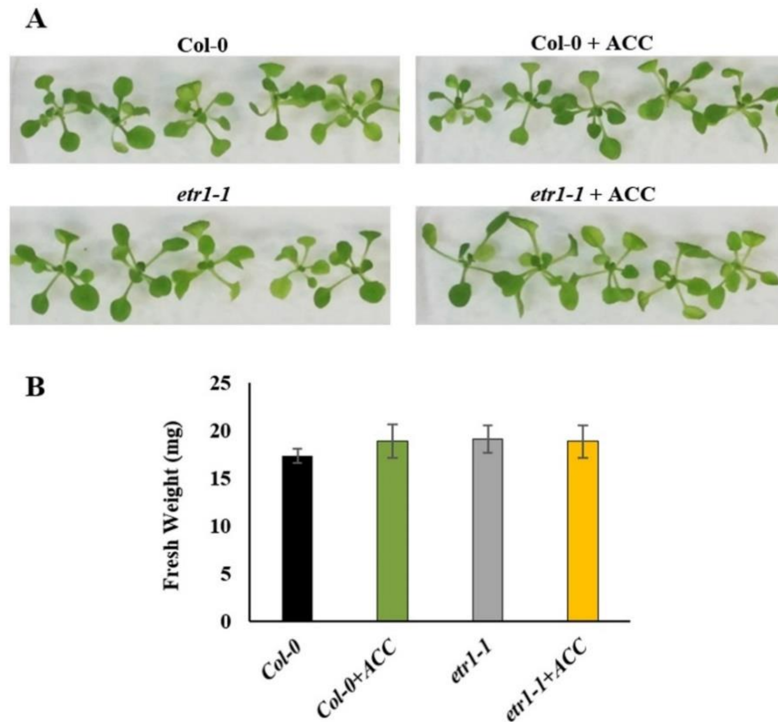
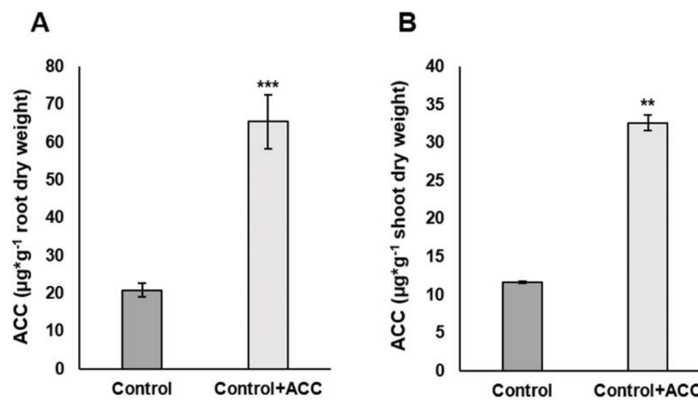
Supplementary data



Supplementary Fig. 1. Effect of ACC on plant growth of *Arabidopsis* seedlings. (A) Representative images and **(B)** shoot biomass production of Col-0 and *etr1-1 Arabidopsis* seedlings 10 days after 100 µM ACC or water treatment are shown. Vertical bars in **B** indicate the calculated standard error of 24 replicates. All values were subjected to analysis of variance, and no statistically significant differences were found.



Supplementary Fig. 2. ACC content in tomato plants after ACC treatment. Roots of three-week-old tomato plants were drenched with either water (Control) or 100 µM ACC. The amount of ACC in **(A)** roots and **(B)** shoots was measured twelve hours after ACC treatment. Vertical bars indicate the calculated standard errors of three replicates (two plants per replicate per treatment). Asterisks indicate statistically significant differences according to Student's *t* test (*$P \leq 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

Supplementary Table 1: Arabidopsis Genome Initiative numbers and primers used in this study for studying expression of Arabidopsis genes.

Name	Sequence[1]	Target	Reference
PR1_F **PR1_R**	5'-TCACAACCAGGCACGAGGAG-3' 5'-CACCGCTACCCCAGGCTAAG-3'	At2g14610, pathogenesis-related gene 1	(Pantelides et al. 2010a)
PR-2_F **PR-2_R**	5'-GCTCTCCGTGGCTCTGACATC-3' 5'-TACCGGAATCTGACACCATCTCTG-3'	At3g57260, β-1,3-glucanase	
PR-4_F **PR-4_R**	5'-ATAATCCGGCGCAGAATAAT-3' 5'-GCGGTCCAGCCATACTTG-3'	At3g04720, hevein-like protein	
PR-5_F **PR-5_R**	5'-GACTCCAGGTGCTTCCCGACAG-3' 5'-ACTCCGCCGCCGTTACATCTT-3'	At1g75040, thaumatin-like protein	
MYC2_F **MYC2_R**	5'-TCATACGACGGTTGCCAGAA-3' 5'-AGCAACGTTTACAAGCTTTGATTG-3'	At1g32640, MYC-related transcriptional activator	
RD22_F **RD22_R**	5'-CTGTTTCCACTGAGGTGGCTAAG-3' 5'-TGGCAGTAGAACACCGCGA-3'	At5g25610, responsive to desiccation 22 gene	
VSP2_F **VSP2_R**	5′-TCAGTGACCGTTGGAAGTTGTG-3′ 5′-GTTCGAACCATTAGGCTTCAATATG-3′	At5g24770, vegetative storage protein 2	
ERF1_F **ERF1_R**	5'-GAAATTCGCGGCGGAGATAAGAG-3' 5'-AACGCCACAACCGGAGAACAAC-3'	At3g23240, ethylene response factor 1	This study
PR3_F **PR3_R**	5'- CAGACTTCCCATGAAAC-3' 5'- TTGGTCCTCTTCCGTA-3'	At3g12500, basic chitinase	(Lee et al. 2009)
MYB51_F **MYB51_R**	5'- ACAAATGGTCTGCTATAGCT-3' 5'- CTTGTGTGTAACTGGATCAA-3'	At1g18570, Indole-Glucosinolate Biosynthesis Transcription Factor	(Millet et al. 2010)
PP2AA3_F **PP2AA3_R**	5'- TAACGTGGCCAAAATGATGC-3' 5'- GTTCTCCACAACCGCTTGGT-3'	At1g13320, Protein Phosphatase 2A Subunit A3	(Czechowski et al. 2005)
MYB72_F **MYB72_R**	5'-ACGAGATCAAAAACGTGTGGAAC-3' 5'- TCATGATCTGCTTTTGTGCTTTG-3'	At1g56160, MYB domain protein 72 transcription factor	(Zamioudis et al. 2015)

Chapter 4

Summarizing Discussion

Plants are capable to modulate the levels of various hormones they produce when exposed to abiotic and biotic stresses such as drought, salinity, heavy metal contamination and pathogen attack. Ethylene production is an early response of plants to perception of such external stimuli and has been associated with the induction of plant defense mechanisms (Abeles et al. 1992; Van Loon et al. 2006). However when ethylene is produced more than its threshold level, it contributes to stress alleviation leading to senescence, chlorosis, leaf abscission and finally growth inhibition (Singh et al. 2015; Van Loon et al. 2006). In the last decade, intense studies of plant growth promoting rhizobacteria (PGPR) revealed an array of beneficial traits they possess not only to stimulate plant growth but also to help plants overcome the toxic effects of their stressors. PGPR are able to stimulate plant growth via the production of phytohormones (e.g. auxins, cytokinins and ethylene) (Persello-Cartieaux et al. 2003), the enzymatic activity they display (e.g. ACC deaminase) (Penrose and Glick 2003b) or by facilitating nutrient uptake, e.g. phosphate solubilization (Rodríguez and Fraga 1999). PGPR can also protect plants against soil-borne plant pathogens by producing antibiotics, hydrogen cyanide (HCN), and siderophores (Handelsman and Stabb 1996; Thomashow 1996; Whipps 2001; Bloemberg and Lugtenberg 2001), or through the activity of fungal cell wall degrading enzymes (Dunne et al. 1997; Chernin et al. 1995). One of the best documented functions of PGPR is the cleavage of ACC via the enzyme ACC deaminase (ACCd). Certain PGPR that possess this enzyme can reduce the levels of the so called "stress ethylene", by breaking down ACC, the immediate ethylene precursor, to α-ketobutyrate and ammonia resulting in a decrease of plant ethylene (Honma and Shimomura 1978; Glick et al. 1998). As a result, plants which grow in association with ACC deaminase-containing PGPR form longer roots and shoots and are more resistant to growth inhibition by a variety of ethylene-inducing stresses (Glick 2014).

On the other hand, several plant pathogenic fungi and bacteria are capable of producing ethylene as a virulence factor, which improves their ability to colonize plant tissues while others can modulate plant ethylene levels to overcome ethylene-induced defense responses (Tzeng and DeVay 1984; Weingart et al. 2001; Cristescu et al. 2002; Al-Masri et al. 2006; Zamioudis and Pieterse 2012). The symptoms of Verticillium wilt disease, caused by the soil-borne fungus *Verticillium dahliae,* have been firmly correlated with symptoms caused upon ethylene exposure. Plants inoculated with *V. dahliae* were found to produce high amounts of ethylene and the symptoms of the

disease were enhanced after ethylene application in infected plants (Cronshaw and Pegg 1976; Pegg and Cronshaw 1976; Tzeng and Devay 1985). Thus it was hypothesized that ethylene production by *V. dahliae* may be used by the fungus to facilitate infection. The genome of *V. dahliae* contains genes encoding ACC synthase, ACC deaminase, and Ethylene Forming Enzyme (EFE), however, it still remains unknown whether the ethylene produced by the fungus is a virulence factor. In addition, mutational studies elucidating the role of these genes in ethylene biosynthesis in fungi are still lacking.

The initial aims of this thesis were to investigate whether *V. dahliae* produces ethylene via the ACC-mediated pathway and whether alterations in ACC content and ethylene production by the fungus could affect its pathogenicity. To accomplish this goal, the endogenous *ACCd* gene of *V. dahliae* was deleted and overexpressed and the resulting mutants were evaluated for their ACC content, ethylene production and pathogenicity (**Chapter 2**). The interesting results obtained by *ACCd* manipulation and particularly the effect of *ACCd* mutants on the ACC content in the roots of infected plants were the driving force to study the potential role of ACC as a potential regulator of virulence or plant defense. Consequently, the second part of this thesis (**Chapter 3**) was devoted to the investigation of the involvement of ACC in the induction of plant defense responses and whether these responses occur in an ethylene independent way.

In this chapter, the main findings of the thesis are connected and discussed with the aim to understand the role of ACC deaminase and ACC in the outcome of the infection of plants by *V. dahliae*.

An alternative role for ACC deaminase in the soil-borne pathogen *V. dahliae*

Plants treated with ACC deaminase-producing PGPR have been shown to be less susceptible to a range of pathogenic agents including pathogenic nematodes, bacteria, fungi and oomycetes (Wang et al. 2000; Saleem et al. 2007; Toklikishvili et al. 2010; Husen et al. 2011; Amutharaj et al. 2012; Nascimento et al. 2013). ACC deaminase-producing PGPR reduce the amount of plant ACC and consequently the “stress ethylene” produced upon pathogen attack (Glick et al. 1998; Glick 2014). The *ACCd* gene is also present in some plant pathogenic bacteria, however it is not clear whether the presence of *ACCd* in these phyopathogens can mask their pathogenic effects and promote plant growth (Saleem et al. 2007; Nascimento et al. 2018).

Exploiting this feature of ACC deaminase-containing PGPR, *ACCd* mutants of *V. dahliae* were generated with an aim to alter the ethylene amounts produced by the fungus and as a result to affect its virulence. Since ACC deaminase cleaves ACC into α-ketobutyrate and ammonia, it was hypothesized that if the ACC-dependent pathway in *V. dahliae* mainly produces ethylene, overexpression of *ACCd* would lead to decreased ethylene production and thereby low virulence while its deletion would increase ethylene production and aggravate disease symptoms. However the results obtained in **Chapter 2** did not support this hypothesis. *ACCd* overexpression in *V. dahliae* led to enhanced virulence in tomato and eggplant while *ACCd* disruption resulted in impaired symptom development in both host plants tested (Fig. 1A). Similar patterns in pathogenicity were observed when the *ACCd* mutants were tested in different growth conditions. Tomato plants infected with *ACCd* deletion mutants displayed less severe symptoms than plants inoculated with the wild type strain or the *ACCd* overexpression strains under greenhouse and hydroponic growth conditions. In addition, *ACCd* overexpression strains exhibited increased *in planta* proliferation since they colonized in a greater extend the vascular system of tomato plants compared to the wild type and *ACCd* deletion mutants. Interestingly, these phenotypes observed could not be attributed to ethylene production, since both types of mutant produced more ethylene than the wild type strain. This indicated that the ACC-dependent pathway may only play a minor role in ethylene biosynthesis in *V. dahliae* and probably ACC must have a different metabolicfunction in *V. dahliae*. However, the possibility that the ACC-mediated pathway could function as a back-up biosynthetic pathway for ethylene production or that ACC, α-ketobutyrate, ammonia, or combinations of these metabolites might affect the production of ethylene via other pathways cannot be excluded. In fact, in some phytopathogenic fungi, such as *Penicillium digitatum* and *Fusarium oxysporum*, more than one ethylene biosynthetic pathways seem to coexist and alternative routes for ethylene production could be triggered according to the available metabolic supply or upon external stimuli (Billington et al. 1979; Fukuda et al. 1986; Hottiger and Boller 1991; Jia et al. 1999; Chagué 2010).

As it was anticipated the manipulation of the *ACCd* gene in *V. dahliae* altered the ACC content of the pathogen; ACC concentration in *ACCd* overexpression strains was significantly lower than that in wild type strain, whereas ACC levels in deletion mutants were higher than that in overexpression strains and the wild type as well. The increased

virulence of *ACCd* overexpression strains in combination with their low ACC content indicated that *V. dahliae* may use *ACCd* to regulate its own ACC levels which could interfere with plant defense during the infection process. It is also possible that *V. dahliae* may use *ACCd* gene to reduce plant ACC and ethylene levels in an effort to overcome the ethylene-regulated plant defense responses and facilitate infection (Fig. 1B). In support of this hypothesis, Guan et al. (2015) demonstrated that plants with reduced ACC production were colonized to a greater extent by the plant pathogen *Pseudomonas syringae*.

Results obtained from **Chapter 2** suggest that the *ACCd* gene of *V. dahliae* is involved in virulence and ACC regulation of the pathogen. Manipulation of the gene pointed out the role of ACC as a potential negative regulator of virulence since the less virulent *ACCd* deletion mutants had a higher ACC content compared to *ACCd* overexpression strains. Consequently, in **Chapter 3** the possibility that ACC accumulation in the root system of the plants could lead to enhanced resistance against *V. dahliae* was further investigated as an alternative scenario that could explain the phenotype observed in the pathogenicity experiments.

The role of ACC in plant infection by *V. dahliae*. Ally or enemy?

Recent studies suggested that ACC could be more than just the precursor of ethylene and act as a signaling molecule regulating plant development and defense responses (Xu et al. 2008; Tsuchisaka et al. 2009; Tsang et al. 2011; Yoon and Kieber 2013; Van de Poel and Van Der Straeten 2014).

In **Chapter 2** it was concluded that ACC seems to act as potential negative regulator of virulence in *V. dahliae.* It was also suggested that *ACCd* overexpression strains might reduce plant ACC levels with the aim to overcome plant defense responses and facilitate infection. In **Chapter 3** the possibility of ACC acting as positive regulator of plant defense against *V. dahliae* was investigated. Consistent with the role of ACC deaminase in cleaving ACC, it was initially shown that *ACCd* overexpression strains decreased ACC levels in the roots of infected plants while in plants infected with *ACCd* deletion mutants ACC levels were significantly higher than those infected with the wild type or overexpression strains. Moreover, external application of ACC to the roots enhanced the resistance of tomato and *Arabidopsis* plants against *V. dahliae,*

strengthening the hypothesis that ACC accumulation in the roots could affect the outcome of the disease to the benefit of the plant.

Foliar application of ACC to *Arabidopsis* and tomato plants has been previously shown to enhance resistance against *Pseudomonas syringae* pv. *tomato* and *V. dahliae,* respectively, but these defense responses were attributed to ethylene rather than ACC (Pieterse 1998; Van Wees et al. 1999; Pieterse et al. 2000; Robison et al. 2001a). However, since in this study ACC was root drenched and not sprayed on the foliage, it seems highly likely that root application of ACC triggered responses similar to those caused during root cell elongation after ACC treatment (Fig. 1D). In this process ACC induces responses such as the production of reactive oxygen species (ROS) leading to hydroxyproline-rich glycoprotein (HPRGs) cross-linking in the cell wall and the quick deposition of callose in the apoplast, contributing to arrest of cell elongation. ACC also induces the down-regulation of cell wall loosening agents like expansins and xyloglucans and the up-regulation of specific cell wall components genes such as HRPGs, auxin related genes and peroxidases that together with their cross-linking enzymes make cells stronger and hence less susceptible to pathogen colonization. (Passardi et al. 2004; Staal et al. 2011; Markakis et al. 2012).

Furthermore, ACC root treatment of *Arabidopsis* and tomato ethylene insensitive plants also led to increased resistance against *V. dahliae*, suggesting that the role of ACC in plant defense could be ethylene independent. To further test this hypothesis, Col-0 and *etr1-1* plants were root treated with ACC in an *in vitro* experiment and it was examined whether ACC could trigger hormone-dependent defense responses (Fig. 1C). In ACC pre-treated Col-0 plants, ET-dependent genes *PR3*, *PR4* and *ERF1* were significantly up-regulated in the roots compared to the untreated controls. Despite the antagonistic relationship of ET and SA, the *PR1* gene was similarly up-regulated in the roots of ACC-treated Col-0 plants, either suggesting that *PR1* induction does not necessarily require SA accumulation or that ACC could trigger plant defense mechanisms independent from ethylene. On the contrary, in ACC pre-treated *etr1-1* plants only *ERF1* was up-regulated despite the enhanced resistance shown by ACC treated *etr1-1* plants against *V. dahliae*. Since *etr1-1* line is ethylene-insensitive and *ERF1* is either induced by JA or/and ET, a possible explanation could be that its up-regulation occurred via activation of JA defense mechanisms without ethylene involvement.

In conclusion, ACC seems to function as a positive regulator of defense, which was suggested by four lines of evidence: (a) *ACCd* deletion mutants which had a higher ACC content than the wild type strain caused significantly less disease symptoms in tomato and eggplant, (b) ACC content in roots of plants infected with *ACCd* deletion mutants was higher than that in mock-inoculated and 70V-WT infected plants, (c) ACC root treatment of plants significantly reduced disease in WT and *Nr* tomato plants and in Col-0 and *etr1-1* Arabidopsis plants and (d) ACC root treatment of Col-0 and *etr1-1* plants *in vitro* induced ET, JA and SA related defense responses.

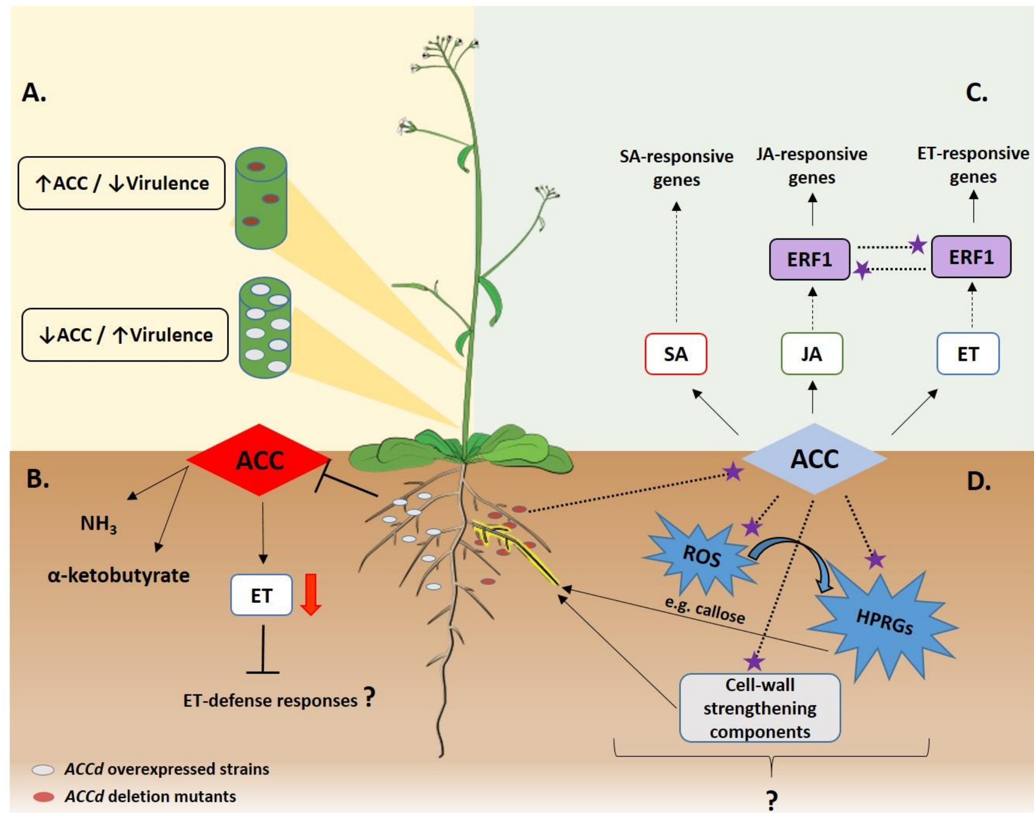


Fig. 1. Schematic representation of the thesis deliverables and the potential mechanisms involved in *V. dahliae* infection by *ACCd* mutants and ACC accumulation in the roots. (A) Effect of *ACCd* and ACC on *V. dahliae* pathogenicity. *ACCd* overexpression strains demonstrate enhanced virulence and *in planta* proliferation and low ACC content indicating the role of fungal ACC as a negative virulence regulator. On the contrary, *ACCd* deletion mutants exhibit high ACC levels but decrease virulence and *in planta* colonization **(B)** Consistent to the function of ACCd, *ACCd* overexpression strains decrease ACC levels in the roots. Impaired ethylene production in the roots could result in the inhibition of ethylene-induced defense mechanisms, allowing *ACCd* overexpression strains to overcome plant defense and facilitate infection **(C)** Exogenous application of ACC in the roots leads to induction of SA-, JA- and ET-mediated plant defense responses in Col-0 and *etr1-1* plants. The derived hypothesis is that *ACCd* deletion mutants which increase the ACC content in the roots of infected plants, could trigger the same plant defense responses as exogenously applied ACC, resulting in impaired disease development. **(D)** Another potential function of fungal ACC or exogenously applied ACC is based on the inhibition of root cell elongation by ACC. ACC accumulation in the roots induces responses such as the production of reactive oxygen species (ROS) leading to hydroxyproline-rich glycoprotein (HPRGs) cross-linking in the cell wall and the quick deposition of callose in the apoplast, contributing to arrest of cell elongation. ACC also induces the down-regulation of cell wall loosening agents and the up-regulation of specific cell wall components genes together with their cross-linking enzymes making cells stronger and hence less susceptible to pathogen colonization. Stars, positive effect; arrows, activation; dashed lines, processes; T-bar, negative effect.

Concluding remarks and future perspectives

In the present thesis, the role of the *ACC deaminase* gene in pathogenicity of *V. dahliae*, ACC content and ethylene production was investigated. In contrast to the plant growth promoting function of ACC deaminase conferred by PGPR, overexpression of *ACCd* in *V. dahliae* resulted in enhanced virulence and colonization of the vascular system of the plants, demonstrating a different role of this gene in phytopathogenic fungi than in the rhizosphere microbiome. Moreover *ACCd* manipulation revealed the role of ACC not only as negative regulator of virulence but also as a potential positive regulator of plant defense. Since it is conceivable that by increasing our understanding of the plant immune system and molecular mechanisms underlying fungal pathogenicity, we can devise novel strategies to reduce crop losses and to enhance plant fitness, future research should focus on the following aspects:

i. Transcriptomic and metabolomic profiling of both *ACCd* overexpression and deletion mutants would be essential to better explain the role of *ACCd* and ACC in the physiology and virulence of *V. dahliae*.

ii. Ethylene determination in plants infected by *ACCd* mutants and transcriptional analysis of defense responses.

iii. Detection of the signaling pathway underpinning ACC triggered defense mechanisms by performing pathogenicity assays using mutant lines of *Arabidopsis* in different hormonal pathways.

iv. Transcriptional and metabolomic profiling of ACC pre-treated plants during infection would also provide significant amount of information.

v. Histochemical observation of the root infection process by *V. dahliae* after ACC treatment would provide essential information about the effect of ACC in the virulence of *V. dahliae* and plant defense responses.

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Research articles in Peer-Reviewed journals:

Tsolakidou, M. D., Pantelides, I. S., Tzima, A. K., Kang, S., Paplomatas, E. J., Tsaltas, D., (2018), Disruption and overexpression of the gene encoding ACC (1-aminocyclopropane-1-carboxylic acid) deaminase in soil-borne fungal pathogen *Verticillium dahliae* revealed the role of ACC as a potential regulator of virulence and plant defense**,** *Molecular Plant-Microbe Interactions,* accepted with minor revision**.**

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Tsolakidou, M. D., Pantelides, I. S., Tzima, A. K., Kang, S., Paplomatas, E. J., Tsaltas, D., (2015), *ACC deaminase* gene is involved in the virulence of the soil-borne pathogen *Verticillium dahliae*, XVIII. International Plant Protection Congress (IPPC), 24- 27 August 2015, Berlin, Germany.

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