Characterization of the virulence of phytopathogenic fungi isolated from a native *Nicotiana attenuata* population

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Van Thi Luu

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First Referee:	Prof. Dr. Rüdiger Hell
	Centre for Organismal Studies, Heidelberg
Second Referee:	Prof. Dr. Sabine Strahl
	Centre for Organismal Studies, Heidelberg
Third Referee:	Prof. Dr. Ian T. Baldwin
	Max Planck Institute for Chemical Ecology, Jena

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Date Van Thi Luu

Contents

1 Abstract

Nicotiana attenuata, a wild tobacco plant, originalted from the Great Basin desert of Utah, USA have been well-studied for its defenses against native herbivores. However, less is known about its natural pathogen community as well as its response against attack from the native pathogens. Two fungal genera comprising three important pathogenic species (*Alternaria alternata* (Fr.) Keissl, *Fusarium solani* (Mart.) Appel and Wollenweber and *Fusarium brachygibbosum* Padwick) were found to cause similar disease symptoms on *N. attenuata* as observed in the native populations of this plant species in Utah 2011. Defined fungal isolates of the three phytopathogen species mentioned above were established as suitable tools to study biological interactions between wild tobacco and its native pathogen community. Moreover, all three main fungal species were demonstrated to be able to induce jasmonic acid (JA), salicylic acid (SA) signaling in *N. attenuata*. Products derived from JA and isoleucyl-JA (JA-Ile) biosynthesis but not JA-Ile perception were shown to play important roles during defense response of *N. attenuata* against the native fungal pathogens. In addition, other compounds, some of which also known for defensive roles such as abscisic acid (ABA), traumatin, dodecenedioic acid (traumatic acid), 9-hydroxy-12-oxo-dodecenoic acid (OH-traumatin), nicotine, rutin, chlorogenic acid (CA), cryptochlorogenic acid (CCA) and dicaffeoylspermidine (DCS) could be shown to be produced in *N. attenuata* after infection of *A. alternata* Utah 10 and/or *F. brachygibbosum* Utah 4. Some JA-inducible secondary metabolites like nicotine and DCS were shown to have big impact on the disease progression caused by Utah fungal pathogens. Among 23 native fungal isolates, *F. brachygibbosum* Utah 10 was the most valuable candidate for the performance of fungal pathogen assays on *N. attenuata* in future. Moreover, this fungus would be also suitable for distinguishing plants in native *N. attenuata* population regarding their traits relevant for pathogen performance and disease progression. Furthermore, an initial investigation of the influence of pre-inoculation with a mutualistic fungus *Piriformospora indica* on the performance of Utah fungal pathogens revealed a benefit for this fungus when *F. brachygibbosum* Utah 4 or *A. alternata* Utah 10 was used to infect *N. attenuata*.

2 Introduction

2.1 Plant pathogens in desert environment

Thinking of foliar plant diseases which occur prequently in temperate climate zones, it's often assumed that they are less likely to appear in arid zones since high humidity often favours the infection process (Wilks and Shen, 1991). This assumption holds partly true for completely arid sites but is erroneous for areas with some forms of moisture such as dew, fog, or rain during specific seasons (Crist *et al*, 1975; Rotem, 1981). For instance, Rotem (1981) reported that some *Alternaria* (Pleosporaceae, Pleosporales, Ascomycota) species even benefit from specific elements of the desert climate, such as drought, wind and sandstorms. It was demonstrated in epidemics caused by *Alternaria solani* Sorauer and *Alternaria alternata* (Fr.) Keissl in the Negev desert, Israel benefit from sandstorms, which maximize spore dispersal, facilitate pathogen entry by generating wounding sites on plants. Furthermore, this infection was favoured by periods of heavy dewfall succeeding sandstorms. Several *Fusarium* species (Nectriaceae, Hypocreales, Ascomycota) grow fairly well in rather dry environments such as *Fusarium solani* (Mart.) Appel & Wollenweber (teleomorph: *Nectria haematococca* Berk. & Broome) which causes dry root rot of beans (Agrios, 2005) and *Fusarium culmorum* (Wm.G. Sm.) Sacc that causes seedling blights of wheats (Colhoun et al., 1968). Apparently that characteristic enables them to cause more severe disease in drier soils, on plants that are stressed by insufficient water (Crist *et al*, 1975). In the desert environment of Arizona, USA, many of the plant parasitic diseases are caused by a limited number of plant pathogens. Most of the important fungal plant pathogens, especially the soilborne fungal pathogens *Fusarium* spp., *Thielaviopsis basicola* and *Macrophomina phaseolina* survive in the soil and cause root crown and wilt diseases on a large number of unrelated plants (Olsen, 1999).

The Great Basin Desert, USA located between the Sierra Nevada Mountains in the west and the Rocky Mountains in the east is the land of extremes in temperature, flora and fauna, landscapes, soil and precipitation. The climate is semi-arid with the majority of the precipitation around 305 mm occurring in spring and winter. Recurring wildfires contribute to the biological diversity of the desert (Mac *et al*., 1998; Pellant *et al*., 2004). Historically, wildfires occurred at return intervals of 32–70 years in sagebrush vegetation types allowing sufficient time in between for the native shrubs, which are generally non-sprouters after a wildfire, to re-establish (Wright *et al*., 1979). The wild tobacco *Nicotiana*

attenuata, a member of the *Solanaceae* plant family can be found in isolated washes that persist for many growing seasons or as an ephemeral component of the postfire annual community in burned sagebrush, blackbrush, and pifiyon-juniper forests of the Great Basin desert. Seeds of *N. attenuata* germinate in the intermediate postfire environment because germination of this plant species is induced by certain cues found in wood smoke (Baldwin and Morse, 1994).

In the summer of 2011, populations of native *N. attenuata* plants in the Great Basin desert of Utah suffered an enormous disease outbreak. Infected plants had diverse symptoms including chlorosis and necrosis of leaves, leaf curling and in some cases also dark spotted patterns on the abaxial leaf surface that looked like sporogenous structures of plant pathogenic fungi (Figure 1). Mapping one of these native *N. attenuata* populations and recording changes in disease progression in a 16 day period revealed that the total number of plants showing disease symptoms slightly increased (around 7 - 9%) (Schuck, S. personal communication). However, the infection process appeared to be incredibly dynamic because many diseased plants recovered either partly or completely, while others got newly developed symptoms. Most of the diseased plants seem to be randomly distributed within the population. Interestingly, diseased plants were often growing right next to healthy plants of the same developmental stage and even having direct physical contact. Moreover, plants of all developmental stages (from early rosette to elongated and flowering plants) were affected. A approximately two weeks before the first survey, strong winds blew sand particles which could damage the plants and provide easy accesses for pathogens. This sandstorm-like event was followed by relatively cool and moist weather conditions (facilitated fungal growth) associated with strong winds (increased risk of pathogen contact). However, it seems that in the vicinity of shrubs and other objects that could have served as a wind shelter, the relative abundance of diseased plants was not lower. These observations suggest that weather, soil and developmental parameters might not be the most important factors determining whether a plant gets infected or not. Rather, genetic variation in plant resistance traits would be a more plausible explanation.

Figure 1: Diseased *Nicotiana attenuata* plants from a native population in the Great Basin desert, Utah, USA. Photo taken by Arne Weinhold.

2.2 Pathogens of solanaceous plants

The *Solanaceae* is a very intriguing family in plant kingdom comprising about 2500 species (Schultes, 1963). Some of them are used for nutrition such as potato (*Solanum tuberosum* L.), tomato (*Solanum lycopersicum* L.), eggplant (*Solanum melongena* L.) and chili pepper (*Capsicum annuum* L.). Because of their high content of alkaloids, some species show interesting pharmacological properties, e.g. Datura (*Datura ssp*.) and deadly nightshade (*Atropa belladonna* L.). The *Solanaceae* family includes also several model plants like *Nicotiana tabacum* (common tobacco), *Nicotiana attenuata* (wild tobacco) and *Solanum lycopersicum* (common tomato) that have been used in many studies.

Fungi are the most common cause of diseases in crops and other plants. More than 10,000 species of fungi can cause diseases in plants. All plants are attacked by some kinds of fungi and each of the parasitic fungi can attack one or many kinds of plants. Some fungi, known as obligate parasites or biotrophs, can grow and multiply only tightly associated with their host plants. Others, known as facultative parasites, require a host

plant for a part of their life cycle but can complete their life cycle on dead organic matter (saprophytically) or they can grow and multiply on dead organic matter as well as on living plants (Agrios, 1998). Solanaceous plants have been found to be infected by many different pathogenic fungi. The common genera of fungal pathogens that infect solanaceous plants are *Alternaria, Fusarium, Phytopthora, Botrytis, Colletotrichum, Verticillium* and *Sclerotinia* (Weber, 1922).

2.2.1 The genus *Alternaria*

Plant diseases caused by *Alternaria* species are very common and distributed all over the world. Important host plants does not only include a variety of crop species such as potatoes, tomatoes, Chinese cabbage, apples but also many ornamental plants and a number of weeds (Franklin, 2001). The genus *Alternaria* contains over 100 species that are mainly saprophytes and commonly found in soil or on decaying plant tissues (Simmons, 1992; Thomma, 2003). *Alternaria* generally attacks the aerial parts of its host, but can also infect roots, tubers, stems and fruits. In leafy vegetables, symptoms of *Alternaria* infection typically start as small, circular, dark spots. As the disease progresses, the circular spots may grow to 1 cm or more in diameter and are usually gray, gray-tan, or nearly black in color. The dying host tissue at the lesion site is also often covered with a fine, black, fuzzy mycelium of sporulating *Alternaria* (Franklin, 2001). Many *Alternaria* species produce toxins that diffuse in host tissues such as altenuene of *A. alternata* and *A. tenuissima* and tenuazonic acid of *A. longipes*, *A. mali* and *A. tenuissima* (Andersen *et al*., 2005; Andersen *et al*., 2008). Therefore it is not uncommon to see a yellow halo that fades into the healthy host tissues surrounding the necrotic lesion spot. *Alternaria solani* is a fungal pathogen that produces a disease in tomato and potato plants called early blight. It can also infect stems, leaves and fruits of eggplant, bell pepper and hot pepper, as well as other members of the *Solanum* genus (Rotem, 1998). Tobacco brown spot is a disease caused by several taxonomically closely related and genetically hardly distinguishable *Alternaria* fungi known as *Alternaria tenuis* Nees, *Alternaria longipes* (El. & Ev.) and *Alternaria alternata* (Fries) Keissl (Spurr and Main, 1974). In living plants, *A. alternata* is an opportunistic parasite, however, it can also penetrate wounded leaves by itself and cause numerous small lesions (Rotem, 1998). In Louisiana, *A. alternata* developed in a complex with *Mycosphaerella gossypina* (G.F. Atk.) and *Cercospora gossypii* Lall, Gill & Munjal. Both fungi are considered saprophytes that colonize plants weakened by low soil moisture, inadequate nutrition, or other pathogens, such as *Xanthomonas campestris pv. malvacearum* (Sinclair and Shatla, 1962).

2.2.2 The genus *Fusarium*

The genus *Fusarium* contains a number of soilborne species with worldwide distribution which have been known for a long time as important plant pathogens, causing various blights, root rots and wilts (Roncero *et al*., 2003). Solanaceous crop plants can be infected at any developmental stage by *Fusarium* fungi which enter the plant vascular system and subsequently use the xylem vessels as avenues to rapidly colonize the host, thereby provoking the characteristic wilt symptoms. *Fusarium spp*. can cause also crown rot and damping-off in tomato, pepper and eggplant. In the sudden death syndrome of soybean caused by *Fusarium solani*, the first leaf symptom to appear is chlorotic mottling, which progresses to interveinal chlorosis and necrosis and defoliation of the leaflets. These symptoms, together with flower and pod abortion, lead to sparse pod development as well as to stunting of plants. In pod lesions usually numerous common saprophytic and weakly parasitic fungi are presented, especially *Epicoccurn nigrum* Link, *Cladosporium herbarurn* (Pers.) Link, and *Alternaria alternata* (Fr.) Keissl. Of these, *A. alternata* was most frequently associated with lesions but it could not be ascertained whether this fungus was the cause of the lesions or was a secondary invader (Melgar *et al*., 1994).

2.3 Effect of phytopathogen communities on their host plants and other interacting partners

In nature, plants are continuously challenged by a community of different pathogens. Indirect interactions between phytopathogenic microorganisms can occur when infection by a first parasite alters the shared host plant in a way that a second parasite is affected which is often spatially or temporally separated from the first one (Mouttet *et al*., 2011). Two pathogen species sharing a common host plant may either compete with or promote each other, or may not affect each other's fitness. For example, infection with biotrophic *Pseudomonas syringae* van Hall rendered *Arabidopsis* plants more susceptible to the necrotrophic pathogen *Alternaria brassicicola* (Schweinitz) Wiltshire (Spoel *et al*., 2007). Pre-infection by *Alternaria alternata* and *Fusarium equiseti* Corda (Saccardo) decreased plant dry weights of maize (*Zea mays* L.), lettuce (*Lactuca sativa* L.) and mycorrhization by *Glomus mosseae* (Nicolson & Gerd.) Gerd. & Trappe (McAllister *et al*., 1997). However, population models of host-parasite interactions predict that when different parasite genotypes compete within a host for limited resources, those that exploit

the host faster will be selected, leading to an increase in parasite virulence (Lopez-Villavicencio *et al*., 2007). Despite their potential importance in predicting the direction and strength of plant-parasite interactions in real ecological communities, studies that focus on natural multiple phytopathogen infections in native populations remain rare.

In the native habitats, *Nicotiana attenuata* is attacked by both bacterial pathogens (*Pseudomonas spp*.) and herbivores (*Manduca sexta*) (Rayapuram and Baldwin, 2008). It also harbors a rich community of endophytic bacteria where *Bacillus sp*. and *Pseudomonas sp*. were the most abundant genera (Long *et al*., 2010). In contrast, this plant species seems to be an unsuitable host for fungi since several tested fungal pathogen species either failed completely or needed a harsh experimental conditions to infect. For example, even by using a very aggressive infection method (needle-prick method as described in Bonaventure *et al*., 2011), *Alternaria brassicicola* and *Botrytis cinerea* hardly caused any disease symptom on the plants (Stefan Schuck, personal communication). Infection during early rosette stage (25 days-old plants) by *Fusarium oxysporum* (f. sp. *lycopersici*) and *Phytophthora parasitica* (var. *nicotianae*) with the needle-prick method worked better and plants developed severe disease symptoms. However most of them died within one week after infection and therefore tiny differences in plant susceptibility towards these pathogens might not observable. Moreover, since *N. attenuata* has been reported to differ a lot in its response to stress or herbivores in natural habitat comparing to glass house (Stitz *et al*., 2011; Đinh *et al*., 2012), it does not seem to be the best to use *P. parasitica* and *F. oxysporum* strains from the "Deutsche Stammsammlung von Mikroorganismen und Zellkulturen" (DSMZ) GmbH, Braunschweig, Germany to explore the plant-fungal pathogen interaction which actually occurs in nature. In this work, fungal pathogens isolated from leaves that were collected from diseased *N. attenuata* plants in Utah, USA, were used to answer the following questions: Which of these fungal pathogens cause similar disease symptoms on *N. attenuata* as observed in native populations? Is any of the Utah phytopathogenic fungal isolates useful for investigation of *N. attenuata*'s response to its native pathogens? How do interactions with other mutualistic fungi affect the disease process?

2.4 Solanaceous plant defenses in response to pathogens

During the last 35 years, a growing body of knowledge on plant-pathogen interactions has been gathered. In general, plants have evolved multiple layers of passive and active defense mechanisms to combat microbial pathogen attack in order to maintain their growth and survival. Passive defense takes advantage of pre-existing structures and pre-formed antimicrobial or toxic secondary metabolites, proteins and peptides. Active defenses, such as oxidative burst, hypersensitive response (HR), accumulation of toxic compounds, and fortification of cell walls, are triggered rapidly and directly in response to pathogen attack (Ding *et al*., 2011).

The molecular mechanisms underlying activation of plant defense responses are exceedingly complex. Responses often begin with gene-for-gene recognition of the pathogen. The production of certain virulence effectors by the pathogen leads to their recognition by plants that carry corresponding resistance genes (*R* genes). Recognition results in rapid activation of defense responses and consequent limitation of pathogen growth. *R* gene-mediated resistance is usually accompanied by an oxidative burst which is a rapid production of reactive oxygen species (ROS). ROS production is required for another component of the anti-pathogen response called hypersensitive cell death in order to limit the access of the pathogen to water and nutrients (Glazebrook, 2005). For example, *Fusarium solani* produces isomarticin and dihydrofusarubin which induce ROS production (Rohnert *et al*., 1998). *R* gene-mediated resistance is also associated with activation of salicylic acid (SA), ethylene (ET) and/or jasmonate (JA) signaling pathways that lead to the expression of certain pathogenesis-related (PR) proteins and the production of antimicrobial secondary metabolites (Glazebrook, 2001). At least 17 families of PR proteins are produced in responses against pathogens (van Loon *et al*., 2006). Most PR proteins are known to possess antimicrobial characteristics. For instance, PR-2 (glucanase) of tobacco has been shown to suppress diseases caused by *Phytophthora megasperma* f. sp. *medicaginins* (van Loon *et al*., 2006). PR-3 (chitinase) isolated from bean can suppress *Rhizoctonia solani* in tobacco (Grover and Gowthaman, 2003). *PR-13*/Na-defensins in *N. attenuata* have been shown to suppress *Pseudomonas syrinage* pv. *tomato* DC3000 (PST DC3000) (Rayapuram *et al*., 2008).

Plant active defensive responses depend on whether the pathogen is biotrophic (feeding on living plant tissue) or necrotrophic (feeding on dead plant tissue). As biotrophic pathogens require a living host, the hypersensitive response which forms localized controlled cell death in the region of pathogen attack is an effective defense

strategy. Necrotrophic pathogens are distinguished from commensals or saprophytes by their ability to actively kill host tissue, therefore programmed cell death initiated by the plant is not an effective strategy to limit necrotrophic pathogen growth (Glazebrook, 2005; Kliebenstein and Rowe, 2008). In general, SA-mediated resistance is effective against biotrophs, whereas JA- or ethylene-mediated responses are predominantly against necrotrophs (McDowell and Dangl, 2000; Glazebrook, 2005). The hemibiotrophic plant pathogen which behaves as a biotrophic pathogen in the early stage of infection but acts as a necrotrophic pathogen in the late stage of colonization induces a complex plant defense response. For example, the resistance of *Nicotiana benthamiana* against the hemibiotrophic pathogen *Phytophthora infestans* requires both ET- and SA-mediated signaling pathways (Shibata *et al*., 2010). Furthermore, Stout *et al*., 2006 proposed that plants strongly control cross-talk between SA- and JA-dependent defenses to prevent adverse signal interactions and maximize their ability to concurrently prevent multiple pathogens.

Chemical defense is provided by secondary metabolites, which can potentially affect all kinds of plant-attacking organisms (Berenbaum 1985). Terpenoids are frequently referred to as natural products active against a variety of herbivores and pathogens including fungi (Litvak and Monson, 1998; Choi *et al*., 2008). *N. attenuata* is known to emit volatile terpenoids when it is attacked by herbivores. These terpenoids have been shown to attract the herbivores' natural predators and to prevent herbivore oviposition (Kessler and Baldwin, 2001). Diterpene glycosides (DTGs) are a diverse group of terpenoid metabolites and hydroxygeranyllinalool (HGL)-DTGs have been isolated from many members of the *Solanaceae*, including *N. attenuata*. High concentrations of those HGL-DTGs effectively defend valuable tissues against herbivores (Heiling *et al*., 2010). Other groups of toxic compound such as alkaloids (nicotine, anabasine and anatabine) and phenolics (rutin, chlorogenic acids and caffeoylputrescine) also play important roles in defense of solanaceous plants against pathogens and herbivores. For example, nicotine, which is produced by members of the genus *Nicotiana*, is effective against herbivores and also exhibits anti-microbial properties (Krischik *et al*., 1991). However, it also has been found to stimulate rust spore germination as well as fungal vacuole formation (French, 1985, 1988). In addition, fatty acids and their derivatives (oxylipins), for example divinyl ethers (DVEs) have been found to be strongly induced in tobacco and potato plants in response to pathogens like *Phytophthora parasitica* var. *nicotianae* and *Phytophthora infestans* (Bonaventure *et al*., 2011). It has been shown that DVEs have antimicrobial

properties of inhibiting mycelial growth and spore germination of some *Phytophthora* species (Prost *et al*., 2005).

During evolution, host plants have developed anti-defense mechanisms that restrict the spectrum of affected species in a way that some secondary metabolites appear to be specialized against particular kind of plant consumers (García-Guzmán and Espinosa-García, 2011). However, pathogens could also evolve to be able to cope with the defensive plant metabolites. For instance, tomato uses α-tomatine against *Septoria lycopersici* which causes leaf spot on this plant (Martin-Hernandez *et al*., 2000). However, some necrotrophic pathogens have evolved the ability to resist α-tomatine by converting α-tomatine to a less toxic derivative, β-tomatine (Quidde *et al*., 1998). Therefore plants typically react with a multi-layer defense response to pathogens. In the responses of wheat to infection by *Fusarium graminearum*, many different genes are induced that involved in various signaling pathways including SA, JA, ET, calcium ion, phosphatidic acid and reactive oxygen species (ROS). Elicitation of these signaling pathways eventually leads to the production of antimicrobial compounds (e.g. phytoalexin camalexin), activation of detoxification mechanisms and cell fortification (Ding *et al*., 2011).

N. attenuata is a model plant for research on the orchestration of plant defenses in nature and the fitness consequences. Therefore *N. attenuata* has been extensively studied regarding to its response to attack by the specialist herbivore *Manduca sexta* (Sphingidae, Lepidoptera) (Baldwin, 2001). In response to damage by *M. sexta, N. attenuata* produces anti-herbivore defense metabolites such as nicotine, caffeoyl putrescine, rutin, and HGL-diterpene glycosides, as well as anti-digestive trypsin protease inhibitors (TPIs) (Rayapuram and Baldwin, 2008). However, much less is known about the response of *N. attenuata* to pathogens, especially to fungal pathogens. By using different fungal pathogens isolated from diseased leaves of *Nicotiana attenuata*, the following questions might be answered: How does the plant respond to those pathogen isolates at molecular level? Can differences in the susceptibility to those isolates be used as a useful tool to distinguish naturally defense-compromised plants in native populations?

3 Aims

Since no natural fungal disease in *N. attenuata* has been reported so far, the main focus of this work was to identify and characterize phytopathogenic fungi isolated from a native *N. attenuata* population as well as to compare the virulence of different isolates. To do so, suitable infection methods for each fungal species needed to be established.

Furthermore, in order to address the defense responses of *N. attenuata* to the Utah pathogenic fungi, phytohormone and secondary metabolite analyses were performed at first, then their contributions to the plant-pathogen interaction has been evaluated by using different transgenic *N. attenuata* plants that were altered in pathogen-inducible phytohormone signaling and secondary metabolite production. By understanding the plant's response to the pathogens, the question of whether or not one can use the Utah fungal isolates as useful tools to distinguish naturally defense-compromised plants within a wild population could be answered.

Being interested in tripartite interactions between the fungi associated with *N. attenuata* plant in nature and the mutualistic fungus *Piriformospora indica*, a multiple fungal infection assay using *P. indica* pre-inoculated plants was performed to see whether or not this fungus could have additive/synergistic effects on the native fungal pathogens. It was also in order to support information for preventing this fungal disease in future by using *P. indica* as a biocontrol agent.

4 Materials and Methods

4.1 Fungi cultivation

Twenty-three Utah fungal cultures were isolated from diseased *Nicotiana attenuata* leaves collected from a native population in the south western Utah, USA. During May and June of 2011, two surveys were conducted on 14 sections of this native *N. attenuata* population comprising 954 plants in an area of about 1500 m^2. Eight leaf samples showing disease symptoms such as curving, chlorosis, necrosis and wilting were collected from different diseased plants within the population and brought to Jena, Germany. Diseased leaves were surface-sterilized in dichloroisocyanuric acid (0.1% DCCS) for two minutes, rinsed thoroughly with sterile distilled water (and briefly dried on sterile filter paper). Afterwards, they were either directly placed in Petri dishes containing potato dextrose agar (PDA) medium (Fluka Analytical, Steinheim, Germany) or ground to obtain leaf suspensions used later for infection of 10 days-old *N. attenuata* seedlings. Seedlings showing disease symptoms were also taken for isolation of pathogens as described above with an incubation time of 1 minute in DCCS. The Petri dishes were kept at 25^{o}C in dark until fungal hyphae emerged from the plant tissue. According to morphological differences, fungal cultures were picked and transferred to new PDA plates. To maintain pathogenicity of certain Utah fungal isolates, the fungi were re-isolated from infected *N. attenuata* plants from time to time and confirmed by morphological comparison to the originally isolates at mycelium growth pattern, structure, color on PDA and conidia-shape under the light microscope.

Piriformospora indica Verma, Varma, Kost, Rexer & Franken (Varma *et al*., 1999) was provided by the department of Plant Physiology from the Friedrich-Schiller University of Jena and was maintained on Kaefer medium (KM; a modified *Aspergillus* minimal medium by Pham *et al*., 2004) containing 1% (w/v) agar.

4.2 Determination of fungal species by internal transcribed spacer (ITS) sequencing

4.2.1 Fungal DNA extraction

14 days-old fungal culture grown on PDA plates was used to extract total fungal DNA according to a modified DNA extraction protocol described for *N. attenuata* by Bubner (2004). Approximately 300 mg fungal mycelium material was scraped directly from the fungal plate's surface and immediately frozen in liquid nitrogen. The samples were ground using a 2000 Geno/Grinder machine (SPEX Certi Prep, Metuchen, NJ, USA). Extraction was performed with 800 µl pre-heated (65°C) buffer (2% CTAB w/v, 100 mM Tris pH 8.0, 20 mM EDTA pH 8.0, 1.4 mM NaCl, 1% PVP M_r 40000, 1% β-mercaptoethanol) and 500 µl of chloroform/isoamyl alcohol (24:1). After mixing and centrifugation at 16,100g for 15 min with Eppendorf centrifuge 5415 R, the supernatant was supplemented with 0.1 volume 10% CTAB (w/v) and further phase-separated by an equal volume of chloroform/isoamyl alcohol (24:1). The nucleic acids in supernatant were precipitated with an equal volume of ice cold isopropanol. The pellet was rehydrated in 500 µl high salt TE (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0, 1 M NaCl, 100 ng/µl RNase A; Roth, Karlsruhe, Germany) and incubated at 37°C for 15 min. Following a phase separation step with 500 µl chloroform/isoamyl alcohols 24:1, the supernatant was taken and the DNA was precipitated by adding 1 ml ice cold 100% isopropanol. The pellet resulting from centrifugation was washed with 80% ethanol and resuspended in 50 µl MilliQ water (Millipore model Milli-Q Advantage A10, Merck, Darmstadt, Germany). OD_{260} measurements on a Biophotometer (Eppendorf, Hamburg, Germany) indicated concentrations between 0.05- 0.25 µg/µl.

4.2.2 ITS region amplification by PCR

A region of internal transcribed spacer (ITS) 1 (about 600 bp), 2 (about 700 bp) and a part of the conserved nuclear ribosomal large subunit (LSU) structural region were amplified by PCR using the primer pair ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA -3') and TW13 (5'- GGTCCGTGTTTCAAGACG -3') (White *et al*., 1990; Gardes *et al*., 1991).

PCR reagents were obtained from Fermentas GmbH, Germany. The PCR mix contained 0.5 µl of a 10 mM deoxynucleoside triphosphate mix (R0191, 0.2 ml), 0.5 µl *Taq* DNA polymerase (EP0403, 100 U), 0.2 µM of each primer, 1 µl 1:10 diluted genomic

DNA extract, 2.5 µl *Taq* buffer (10x), 2 µl $MgCl_2$ (25 mM) and the appropriate amount of Mili-Q water to obtain a final volume of 25 µl.

PCR amplification was conducted on a Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany) with the following program: initial denaturation for 3 min at 95°C, followed by 20 cycles of (i) denaturation for 30 s at 95°C, (ii) annealing for 30 s at 60°C, (iii) elongation for 3 min at 72°C and 72°C for 10 min was setted up for the final extension step. Amplification products were visualized and quantified using horizontal gel electrophoresis with 1% agarose gels (Sigma-Aldrich Chemie GmbH, Hamburg, Germany) in 0.5 M Tris-borate-EDTA-running buffer and ethidium bromide staining.

4.2.3 ITS sequencing

PCR products were sequenced from both strands using the same primers as described above. *BigDye* terminator cycle sequencing (Applied Biosystems, Foster City, USA) was employed as recommended by the manufacturer. All cycle sequencing reactions were performed on a Eppendorf Mastercycler gradient thermocycler using an initial denaturation step at 96°C for 5 min, followed by 35 cycles of 96°C for 10 s, 30°C for 20 s, and 60°C for 4 min. Products were purified using the Big Dye Purification Kit (DyeEx 2.0 Spin KIT, Qiagen http://www.qiagen.com), dried in a vacuum centrifuge and resuspended in template suppression reagent (Applied Biosystems, Foster City, USA). Products were then analyzed on a 16-capillary 3130xl Genetic Analyzer (Life Technologies GmbH (Applied Biosystems Division) Darmstadt, Germany)

4.2.4 Phylogenetic analyses of fungal ITS sequences

The sequences were formatted and edited using Chromas Lite (Technelysium Pty. Ltd., Australia) and Multalin software (http://multalin.toulouse.inra.fr/multalin/). The appropriate sequences were then compared to sequences listed in the GenBank database using the BLASTn program (http://www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 5 (Tamura *et al*., 2011). All fungal sequences are listed in Supplemental Data 1.

The identification of species through sequence similarity was performed according to Stackebrandt and Goebel, 1994: when the percentage of similarity of the query sequence and the reference sequence is 99% or above, the unknown isolate would be assigned to the reference species; when percentage similarity is between 97-99%, the unknown isolate would be assigned to the corresponding genus.

4.3 Fungal morphological characterization by microscopy

14 days-old fungal isolates grown on PDA plates were used for morphological observation. Mycelium and spore mats were scraped from the plate surface and placed on top of microscopy slides with two drops of sterile water. The structures of fungal spore/conida and mycelium were characterized under light microscope (Axio Observer D1, Carl-Zeiss Jena, Germany). Microscopy pictures were taken using AxioVision imaging software at 200 or 400-fold magnification.

The description of *Fusarium solani* by de Hoog (2000) was used for confirmation of *Fusarium solani* Utah, together with reference pictures of its microconidia and macroconidia made by David Geiser (Zhang, *et al*., 2008). *Alternaria* species were characterized using descriptions made by Simmons (1993) together with reference picture of *A. alternata* conidia and conidiophores made by de Hoog *et al*. (2000). Spore dimension of *Fusarium brachygibbosum* was measured at length and width. Spore color and structure was described.

4.4 Fungal metabolic profiling

Fungal metabolite extraction was done using 14 days-old PDA cultures grown at 25°C in darkness. The extraction protocol used was adapted from Andersen *et al*. (2005). Three agar plugs (6 mm diameter) were cut from the center, margin and intermediate area of the fungal colony. The plugs were extracted with 1.0 ml of ethyl acetate containing 1% formic acid by sonication for 60 min. The extract was transferred to a clean 2-ml vial and evaporated in a rotary vacuum concentrator (Eppendorf, Wesseling-Berzdorf, Germany). The residue was redissolved ultrasonically in 200 µl of methanol and centrifuged at 16,100g for 15 min. Two hundred microliters of the supernatant were transferred into HPLC vials.

Fungal metabolites were seperated by Ultraperformance LC-ToF-MS (Agilent 6890N GC (Agilent Technologies, Böblingen, Germany) using a modified version of a previously described LC-ToF-MS method for *N. attenuata* (Gaquerel *et al*., 2010). Four microliters of the fungal extract was injected into a C18 column (Acclaim, 2.2 µm particle size, 150 x 2.1 mm inner diameter (Dionex Corporation, Sunnyvale, USA) and separated using an RSLC system (Dionex). Solvent A was deionized water containing 0.1% (v/v) acetonitrile (Baker, HPLC grade) and 0.05% (v/v) formic acid. Solvent B was acetonitrile

and 0.05% (v/v) formic acid. The gradient condition was applied as follows: 0 to 0.5 min 10% B, 0.5 to 6.5min linear gradient 80% B, 6.5 to 10 min 80% B, and reequilibration at 10% B for 3 min. The flow rate was 300 µl min^{-1}. Eluted compounds were detected with a MicroToF mass spectrometer (Bruker Daltonic, Bremen, Germany) equipped with an electrospray ionization source in positive ion mode. Instrument settings were as follows: capillary voltage: 4500 V; capillary exit: 130 V; dry gas temperature: 200^{o}C; drying gas flow: 8 liters min^{-1}. Mass calibration was performed using sodium formeate clusters (10mM solution of NaOH in 50/50% [v/v] isopropanol/water containing 0.2% formic acid).

The raw data files were converted to netCDF format using the export function of Bruker software (Data Analysis v4.0) and processed using the XCMS package (Tautenhahn *et al*., 2008) and the R-package CAMERA (http://www.bioconductor.org/biocLite.R) as previously described (Gaquerel *et al*., 2010). Peak detection was performed using the centWave method (Tautenhahn *et al*., 2008) and the parameter settings ppm= 20, snthresh = 10, peak width = 5 to 20 s. Retention time correction was achieved using the parameter settings minfrac = 1, bw = 60 s,mzwid = 0.1D, span = 1, andmissing = extra = 0 (Gaquerel *et al*., 2010).

The Metaboanalyst 2.0 online software (http://www.metaboanalyst.ca/MetaboAnalyst/faces/Home.jsp) was used to perform multivariate analysis (PCA and PLSDA). The data was filtered by coefficient of variation, and normalized using Pareto scaling. PLSDA was validated using a permutation test as previously described by Westerhuis *et al*., 2008. An important output of the PLSDA analysis is that it estimates and ranks the influence of individual features (ions) on the model by assigning to each variable a VIP value, and VIP values bigger than or equal to 1.0 are considered statistically significant for group discrimination (Xie *et al*., 2008).

4.5 Plant material and growth conditions

4.5.1 Plant material

Seeds of the 31st generation of an inbred *Nicotiana attenuata* Torr. ex S. Watson, originally collected from south western Utah in 1988, were used for all experiments, and are referred to as "Utah wild type" plants (UtWT). In addition, another *N. attenuata* inbred line (21st generation) originally collected from Arizona and described by Glawe *et al*., 2003 was used for some experiments and is referred to as "Arizona wild type" (AzWT).

All *N. attenuata* transgenic plants used in this study were transformed with endogenous and heterologous genes in sense or antisense/inverted repeat (IR) orientations by *Agrobacterium*-mediated transformation, except for ovNahG plants. aslox3 plants are transformants carrying a plasmid construct encoding *N. attenuata* lipoxygenase 3 (*LOX3*) gene in antisense orientation, this enzyme catalyzes one of the initial steps of jasmonic acid (JA) production in *N. attenuata* plants (peroxidation of trienoic fatty acids at the C_{13} position (Halitschke and Baldwin, 2003)). irgla1, ircoi1, irpmt, and irmyb8 lines were generated by transformation of constructs carrying gene fragments in an inverted repeat orientation: GLA1 cleaves polyunsaturated fatty acids (PUFAs) from chloroplast membrane lipids and provides them as substrate to lipoxygenases (LOXs) (Bonaventure *et al*., 2011); COI1 is involved in JA perception (Paschold *et al*., 2007); PMT is an essential enzyme in nicotine biosynthesis (Zavala *et al*., 2004); MYB8 is a transcription factor mediating herbivory-induced production of phenylpropanoid-polyamine conjugates (PPCs) and other polyamine metabolites accumulation (Kaur *et al*, 2010). A cross (irjar4/6) between two *N. attenuata* lines silenced in either JASMONATE-RESISTANT 4 (*JAR4*) or JASMONATE RESISTANT 6 (*JAR6*) is compromised in the conjugation of JA to amino acids, resulting in low levels of JA-isoleucine (Ile), JA-leucine (Leu), and JA-valine (Wang *et al*, 2008). ovNahG plants are ectopically expressing the bacterial salicylate hydroxylase gene (*NahG*) from *Pseudomonas putida* therfore have low levels of salicylic acid (SA) and they were obtained as described by Gilardoni *et al*., 2011. All transgenic plants were homozygous for a single transgene insertion, diploid as determined by flow cytometry, and used in the T2-T3 generation. All stably transformed lines are listed in Supplemental Table 1 with details of plasmid structures and references.

Seeds of *N. attenuata* native plants were collected from 31 different accessions in Utah and Arizona , USA, from 1998 to 2009 by employees of the Molecular Ecology department, Max Planck Institute for Chemical Ecology, Jena, Germany. All accessions with plant numbers used for detached leaf assay are listed in Supplemental Table 2.

4.5.2 Plant germination and growth conditions

Seeds were germinated on agar plates containing Gamborg's B5 medium as previously described by Krugel *et al*., 2002. Seeds were sterilized for 5 min in aqueous solution containing dichloroisocyanuric acid with 0.1 g/ 5 ml (DCCA: Sigma, St. Louis, MO, USA), supplemented with 50 µl of 0.5% (v/v) Tween-20 (Merck, Darmstadt, Germany). Seeds were washed 3 times with sterile water before incubation for 1 h in sterile liquid smoke (House of Herbs, Inc.; Passaic, New Jersey, USA) solution, 50x diluted in deionized water and supplemented with 50 µl of 0.1 M gibberellic acid, GA3 (Roth, Karlsruhe, Germany). After this treatment, seeds were washed 3 times with sterile water. 25 seeds were transferred individually to a petri dish containing germination medium of Gamborg's B5 with minimal organics and 0.6% (w/v) phytagel (Sigma). Plates were maintained in a growth chamber (Snijders Scientific, Tilburg, the Netherlands) at 26^{o}C for 16 h light (155 µmol s^{-}1 m^{-}2) and 24^{o}C for 8 h dark in a period of 10 days.

Ten days-old *N. attenuata* seedlings which used for inoculation with Utah fungi were transferred to TEKU plastic pots (Pöppelmann GmbH and Co. KG, Lohne, Germany) with sterile sands and grown in climate chamber with a day/night cycle of 16 h (20°C)/8 h (20°C) under supplemental light from Master Son-T PIA Agro 400 and a relative humidity of 60%. After 10 days, early rosette plants were transferred into 10 cm pots and grown in the same climate chamber. Each plant was fertilized with 50ml of $Ca(NO_3)_2$ x $4H_2O$ and 0.4 g/l Flory B1. Plants used for detached leaf assays were transferred after 10 days in the growth chamber into the TEKU plastic pots with Klasmann plug soil (Klasmann-Deilmann GmbH, Geesten, Germany). After 10 days in Teku pots, seedlings were transferred to 1 L pots in soil (0.75 g Superphosphate, Multimix 14:16-18 (Haifa Chemicals Ltd., Haifa Bay, Israel), 0.35 g $MgSO_4x7H_2O$ (Merck KGaA, Darmstadt, Germany) and 0.05 g Micromax (Scotts Deutschland GmbH, Nordhorn, Germany) per 1 L of Fruhstorfer Nullerde (Hawita GmbH, Vechta, Germany)). Fertilization was done also by flood irrigation with addition of 3.0 g Borax (Nic Sosef BV, Honselersdijk, Netherlands) on the day of potting; 2.0 g Borax and 20 g Peters Allrounder 20:20:20 (Scotts International, Heerlen, Netherlands) from day seven on; 1.0 g Borax, 40 g Peters Allrounder from day 14 on; and 25-30 g Peters Allrounder from day 21 on; each to a 400 L watering tank. From day one to day five, plants were watered with 300 ml and later with 100 ml/d and grown in the glasshouse at 26-28ºC under 16 h supplemental light from Master Sun-T PIA Agro 400 or Master Sun-T PIA Plus 600 W Na lights (Philips, Turnhout, Belgium).

4.6 Detached leaf assays

A detached leaf assay was conducted for testing pathogenicity of Utah fungal isolates and primarily screen for the differences between *Nicotiana attenuata* accessions in their resistances to one of the Utah fungal isolates.

Fully expanded leaves were collected from plants at rosette stage (30 days-old plants). The leaves were placed upside down on four layers of moist-autoclaved tissue paper in square-shaped petri dishes. Plugs of fungal mycelium (4 mm diameter) were cut from the edge of actively growing colonies culured on PDA for 14 days. Three fungal agar plugs were placed on three different spots of a detached *N. attenuata* leaf. All plates were sealed twice with paraffin to maintain moist conditions (> 90% relative humidity) and kept at 25°C with 14 hours photoperiod. After incubation, the diameter of chlorotic and necrotic lesions was measured until they overlaped too much or the leaves showed senescent.

Detached leaf assays were carried out with 12 biological replicates per isolate for testing pathogenicity of Utah fungal isolates.

For the detached-leaf assay with different *N. attenuata* accessions, 99 plants were randomly picked from 31 different accessions (see plant numbers and accessions in the Supplemental Table 2). One leaf per plant was used with three replicates per leaf. Diameter of hypersensitive response lesions (indicator for induced resistance) and chlorotic lesions (resembling rather basal resistance) were measured at 3, 4 and 5 days after inoculation. Percentage of hypersensitive response and chlorosis was then multiplied to obtain a "product" value. This product value was used to evaluate the response of each accession using the following categories: "Product" ≤ 247: Hyper-resistance (R^+); 247 < "Product" ≤ 728: Resistance (R); 728 < "Product" ≤ 2384: Indifferent (Ind); 2384 < "Product" ≤ 3930: Susceptible (S); "Product" > 3930: Hyper- susceptible (S^+).

4.7 Infection of Utah fungi on intact *N. attenuata* plants

4.7.1 Spore harvesting

The inoculum consisted suspension of spores that were washed from the surface of fungal plates after 2 weeks grown at 24^{o}C in dark. Spores were filtered through miracloth (Calibiochem, UK) to remove mycelial fragments and centrifuged at 800g for 20 min. The pellets were washed twice with 10mM $MgSO_4$ and the spore concentration was adjusted by using a Neubauer hemocytometer to count the number of spores under the light microscope.

4.7.2 Screening for a suitable infection method

In order to find out suitable infection methods for the main Utah fungal species, screening assays were performed using Utah fungal isolates with different pathogenicity (most aggressive and moderate isolates as determined by detached leaf assay) and three different spore concentrations (10^5, 10^6 and 10^7 spores per milliliter). Those fungal isolates were infected to *N. attenuata* plants of different age (from 10 days-old or 20 days-old) using two different infection methods: root-dip and spraying. For root-dip method, roots were dipped in spore suspension for 30 s and afterwards placed back into the substrate. Spraying with spore suspension was performed homogenously from a 20 cm distance to the plant with approximately 0.3 ml spore suspension per seedling. Control plants were dipped into or sprayed with 10mM $MgSO_4$ solution.

In addition, to find out an infection method which is also suitable for different genotypes of *N. attenuata*, aslox3 and ircoi1plants which are compromised in JA biosynthesis or signaling were included in this screening assay.

4.7.3 Fungal infection proceduce

Infection of certain Utah fungal isolates individually was done according to results from the screening assays mentioned above. In order to optimize the infection protocol, the infection assay was repeated for *A. alternata* Utah 10 and *F. brachygibbosum* Utah 4 with slightly modified experimental design. All of these changes are listed below for each fungal infection assay.

* *Alternaria alternata* Utah 10 infection:

First infection assay: 10 days-old *N. attenuata* seedlings germinated on GB5 plates were transplanted into Φ3 cm plastic pots and placed in glasshouse (26-28ºC under 16 h

supplemental light and 8h dark) for 5 days before the infection. Infection was done by root-dip method with 10^5 spores/ml. After infection, plants were placed in climate chamber (20°C, 16 h light and 8 h dark, 60% humidity). After 5 days when plants were 20 days-old, they were transplanted into Φ10 cm plastic pots. Plants dipped in 10 mM MgSO4 solution were used as controls (non-infected plants). 20 biological replicates were used for one treatment.

Second infection assay: 10 days-old *N. attenuata* seedlings germinated on GB5 plates were transplanted into Φ3 cm plastic pots and placed in climate chamber (20°C, 16 h light and 20°C, 8 h dark, 60% humidity) for two days prior to infection. Infection was done by root-dip method with 10^5 spores/ml. At 8 days after infection, 20 days-old plants were transplanted into Φ10 cm plastic pots. Plants dipped in 10 mM MgSO4 solution were used as controls (non-infected plants). 25 biological replicates were used for one treatment.

* *Fusarium brachygibbosum* Utah 4 infection:

First infection assay: 10 days-old *N. attenuata* seedlings germinated on GB5 plates were used directly for infection by dipping seedling roots into a suspension of 10^7 spores/ml. Plants were then grown into Φ3 cm plastic pots and placed in climate chamber (20°C, 16 h light and 20°C, 8 h dark, 60% humidity). After 10 days, when plants were 20 days-old, they were transplanted into Φ10 cm plastic pots. Plants dipped in 10 mM $MgSO_4$ solution were used as controls (non-infected plants). 20 biological replicates were used for one treatment.

Second infection assay: 8 days-old *N. attenuata* seedlings germinated on GB5 plates were transplanted into Φ3 cm plastic pots and placed in a climate chamber (20°C, 16 h light and 20°C, 8 h dark, 60% humidity) for two days prior to the infection. Infection was done by root-dip method with 10^7spores/ml. At 10 days after infection, 20 days-old plants were transplanted into Φ10 cm plastic pots. Plants dipped in 10 mM $MgSO_4$ solution were used as controls (non-infected plants). 25 biological replicates were used for one treatment.

* *Fusarium solani* Utah 4 infection:

10 days-old *N. attenuata* seedlings germinated on GB5 plates were transplanted into Φ3 cm plastic pots and placed in glasshouse (26-28ºC under 16 h supplemental light and 8 h dark) for 10 days prior to infection. Infection was done by root-dip method with 10^5 spores/ml. After the infection, plants were grown in Φ10 cm plastic pots in climate chamber (20°C, 16 h light and 20°C, 8 h dark, 60% humidity). Plants dipped in 10 mM

$MgSO_4$ solution were used as controls (non-infected plants). 20 biological replicates were used for one treatment.

* *Piriformospora indica* preinoculation: *N. attenuata* seeds were germinated on GB5 plates which were pre-inoculated for two weeks with 14 days-old *P. indica* culture. Until then, the *P. indica* pre-inoculated GB5 plates were placed in the same conditions as described for *N. attenuata* seed germination.

* Multiple fungal infection assay of *N. attenuata* with *P. indica* and two Utah fungal isolates: 12 days-old *N. attenuata* seedlings germinated on *P. indica* pre-inoculated GB5 plates were used for infection with either *F. brachygibbosum* Utah 4 or *A. alternata* Utah 10 by using infection proceduces described above for the second infection assay of each. Plants dipped in 10 mM $MgSO_4$ solution were used as controls of *P. indica* pre-inoculated plants. The experimental design is described in Supplemental Figure 1. Twenty to twenty-five biological replicates were used for each infection experimental group.

4.8 Symptom quantification and plant morphology

Disease symptoms were recorded from 5 days after infection (dai) on. Disease rating was based on the percentage of chlorotic, necrotic and wilting symptoms.

Rosette diameter was measured daily starting at 1 dai and every 3 days after 20 days of infection. About 1 month after germination, when plants had reached the elongation stage, stalk length was measured every third to fourth day and the time of bolting (appearance of the floral meristem) and flowering was recorded for each plant. When plants stopped elongation (about 60 days after germination), final stalk length was measured. Flower morphology was also observed during flowering time.

4.9 Isolation of genomic DNA and quantification of fungal DNA in infected leaves by quantitative real time PCR (qPCR)

For quantification of fungal colonization rates, DNA was extracted from 100 mg homogenized plant material according to the CTAB method described by Bubner *et al*. (2004), modified for PCR purpose. In principle, the samples were extracted in 750 µl 2% CTAB hot (65°C) buffer (2% CTAB w/v, 100 mM Tris pH 8.0, 20 mM EDTA pH 8.0, 1.4 mM NaCl, 1% PVP M_r 40000, 1% β-mercaptoethanol) and 750 µl of chloroform/isoamyl alcohol 24:1. After being mixed and centrifuged at 16,100g for 15 min, the supernatant was incubated with 0.5µl RNase A at 37°C for 20 min and again phase-separated with 750

µl chloroform/isoamyl alcohol 24:1. The nucleic acids in the resulting supernatant were precipitated with 700µl ice cold isopropanol. The pellet resulting from centrifugation was washed with 75% ethanol and the final DNA pellet was dissolved in 50µl MiliQ water. OD_{260} measurements on a Biophotometer (Eppendorf, Hamburg, Germany) indicated concentrations between 0.5–1.2 µg/µl. All samples were obtained by pooling of three biological replicates.

15 ng DNA was used for quantitative PCR, which was performed with a Mx3005P Multiplex qPCR system (Stratagene, La Jolla, CA) and the qPCR Core kit for SYBR® Green I (Eurogentec, Liege, Belgium). Relative quantification of fungal genomic DNA was performed by the comparative cycle threshold (CT) method using the eukaryotic elongation factor 1A (NaEF1A) gene as a reference. All the reactions were performed using the following qPCR conditions: initial denaturation step of 95^{o}C for 30 s, followed by 40 cycles each of 95^{o}C for 30 s and 60^{o}C for 1min, with a final extension step of 95^{o}C for 30 s and 60^{o}C for 1 min (Gilardoni *et al*., 2010).

Specific primers of *A. alternata*, *F. brachygibbosum* Utah 4 and *P. indica* have respective sequences: alt-F3 (5'-TCTAGCTTTGCTGGAGACTC-3') and alt- R1.1 (5'-AGACCTTTGCTGATAGAGAGT- 3') (Schuhegger *et al*., 2006); F. bra-F (5'-GATCGGGCTGTACTCCAG-3') and F. bra-R (5'-CATTCAGAAGTTGGGGGTTTAAC-3')(designed, derived from ITS sequences); Piri ITS-F 5'-CAACACATGTGCACGTCGAT-3' and Piri ITS-R 5'-CCAATGTGCATTCAGAACGA-3'(derived from sequence of translation elongation factor 1a (Pi-EF1A) gene) (Deshmukh *et al*., 2006). The primers were checked beforehand for their specific by normal PCR using genomic DNA of those fungi, *N. attenuata* and other fungal species, including *P. indica*, *Phytophthora parasitica* var. *nicotianae* and *Sebacina vermifera* as templates.

4.10 Phytohormone extraction and quantification by LC-ESI-MS/MS

50-100 mg of frozen entire seedling material was homogenized to a fine powder with a Geno/Grinder 2000 (BTC and OPS Diagnostics, Bridgewater, USA). Five hundred micro-milliliter of ethylacetate spiked with 100 ng of [$^{2}H_{2}$]JA, 20 ng of [$^{2}H_{4}$]SA, 20 ng of ABA and 20 ng of JA-Ile was added to each sample. After thoroughly mixing, they were centrifuged for 15 min at 12,000g (4^{o}C). The upper organic phase was transferred into a fresh tube, and the leaf material was re-extracted with 0.5 mL ethylacetate without internal

standards. The organic phases were pooled and evaporated to dryness under reduced pressure. The dry residue was reconstituted in 0.4 mL of 70/30 (v/v) methanol/water.

Measurements were conducted on a LC-ESI-MS/MS instrument (Varian 1200 Triple-Quadrupole-LC-MS system; Varian, Palo Alto, CA). Ten microliters of the sample were injected in a ProntoSIL column (C18-ace-EPS, 50 x 2 mm, 5 µm, 120 A^o; Bischoff, Leonberg, Germany) connected to a precolumn (C18, 4 x 2 mm; Phenomenex, Aschaffenburg, Germany). As mobile phases, 0.05% formic acid in water (solvent A) and methanol (solvent B) were used in a gradient mode with the following conditions: time/concentration (min/%) for B: 0.0/15; 2.5/15; 4.5/98; 10.5/98; 12.0/15; 15.0/15; time/flow (min/mL min^{-1}): 0.0/0.4; 1.5/0.2; 1.5/0.2; 10.5/0.4; 15.0/0.4. A negative electrospray ionization mode was used for detection. An ion with a specific mass-to-charge ratio generated from each endogenous phytohormone or internal standard (the parent ion) was selected and fragmented to obtain its daughter ions; a specific daughter ion was used for generating the corresponding compound's chromatogram. Each phytohormone was quantified by comparing its peak area with the peak area of its respective internal standard. The method was developed by Wu *et al*., 2007. Divinyl ethers were quantified using the method described by Bonaventure *et al*. (2011). During the first infectin assay, all phytohormone levels were expressed in nmol per gram fresh weight. Since some seedlings showed wilting symptoms, the phytohormone levels were referred to dry weight to avoid inaccuracies in the second infection assay.

4.11 Secondary metabolite extraction and measurement by HPLC

For analysis of secondary metabolites in fungi-infected and non-infected *N. attenuata* plants, five biologically replicates with 2 seedlings pooled for each replicate were harvested at three different time points.

Nicotine, dicaffeoyl spermidine (DCS), chlorogenic acid (CGA), cryptochlorogenic acid (Cryto CGA) and rutin were analyzed using an HPLC-DAD method (Keinanen *et al*., 2001). 50-100 mg of entire seedling material were homogenized with 2 steel balls by Genogrider 2000 (SPEX Certi Prep) at 1200 strokes per minute for 30 seconds and then extracted with 1 mL extraction solution (40% methanol, 0.5% acetic acid) per 100 mg sample fresh weight. After centrifugation at 16,100g for 20 min at 4^{o}C, the supernatants were injected into an Agilent 1100 HPLC (Agilent HPLC 1100 Series, Palo Alto, CA) installed with a Chromolith FastGradient RP-18 (5032 mm; Merck,

Darmstadt, Germany) endcapped 50 x 2 mm HPLC column (Lot No. HX802433 Merck, Darmstadt, Germany) attached to a Gemini NX RP18, 3µm, 2 x 4.6 mm precolumn (Phenomenex, Aschaffenburg, Germany) with a column oven set at 40°C. The mobile phase consisted of a mix of solvent A (0.1 % formic acid and 0.1 % ammonium hydroxide solution in water (pH 3.5) and solvent B (methanol) was used in a gradient mode (time/concentration min/% for A: 0:00/100; 0.50/100; 6.50/20; 10:00/20; 15:00/100) with a flow rate 0.8 mL/min. Under these conditions, nicotine eluted at a retention time (RT) of 0.5 min (detected by UV absorbance at 260 nm); chlorogenic acid, cryptochlorogenic acid and dicaffeoyl spermidine (DCS) eluted at RTs of 3.1, 3.5, and 4.0 min, respectively (detected at 320 nm). Rutin eluted at RT 4.7 min and it was detected at 360 nm. The peak areas were integrated using the Chromeleon chromatographic software (version 6.8; Dionex, Sunnyvale, USA) and the amount of metabolites in plant tissue was calculated using external dilution series of standard mixtures of nicotine, chlorogenic acid and rutin. Crypto-chlorogenic acid and DCS contents were estimated based on the external calibrations. The method was described previously by Kaur *et al*. (2010)

4.12 Statistical Analysis

Statistics were performed using the SPSS software version 17.0 (www.spss.com). Statistical significance was evaluated using one-way analysis of variance (ANOVA) at a 0.05 level and means were compared by the LSD post-hoc test. For analysis of differences in plant performance the *t* test was used with the one-tailed distribution of two sets of samples with equal variance. The number of replicates (*n*) used in each experiment are detailed in the Figure captions.

5 Results

5.1 Identification of fungi species from Utah by ITS sequencing

The amplification of the ITS region including ITS1, 5.8S and ITS2 sequences plus a part of the large ribosome subunit sequence resulted in a range from 1090 to 1178 bp depending on fungal isolate. For the fungal isolate from Utah number 17 (Utah fungal isolate 17), only a 585 bp long ITS region could be amplified and used for sequencing. All fungal DNA sequences comprising ITS sequences are listed in Supplemental Data 1.

The BLASTn of the Utah fungal isolates 1 to 4 revealed 99% sequence homology to *Fusarium brachygibbosum* strain NRRL 34033 (GenBank accession number GQ505450.1). Therefore these four Utah fungal isolates were assigned to *F. brachygibbosum* Utah 1, *F. brachygibbosum* Utah 2, *F. brachygibbosum* Utah 3 and *F. brachygibbosum* Utah 4. Six Utah fungal isolates (number 5 to 10) had 99% sequence similarity to *Fusarium solani* strain ATCC 56480 (GenBank accession number FJ345352.1). Those isolates therefore were renamed to *F. solani* Utah 1, *F. solani* Utah 2, *F. solani* Utah 3, *F. solani* Utah 4, *F. solani* Utah 5 and *F. solani* Utah 6. The ITS plus LSU sequence of the Utah fungal isolate number 11 was showing 99% homology with *Fusarium sp.* (gb.HQ130706.1) and 98% similarity to *Fusarium oxysporum* f. sp. *rapae* (GenBank accession no: AB586994.1). Following the criteria of identification as described in materials and methods, the Utah fungal isolate number 11 was classified at genus level as *Fusarium sp.* Utah 1. The BLASTn result for 12 other Utah fungal isolates (numbers 12 to 23) revealed 99% sequence similarity to *Alternaria sp*.(AY154708.1/ AY154693.1/ GU187964.1/ HM751087.1), *Alternaria alternata* strain SS-L6 (GenBank accession no: GU797144.1), *Alternaria tenuissima* strain IA287 (GenBank accession no: AY154712.1), *Alternaria longipes* (GeneBank accession no: AY154684.1) and *Alternaria mali* (GeneBank accession no: AY154683.1), indicating that these 12 Utah fungal isolates belong to the genus *Alternaria* and they are indistinguishable at species level by ITS and LSU sequence comparison.

The phylogenetic tree was constructed using Mega5 software (neighbor-joining (NJ) tree using Kimura-2-parameter with 1000 bootstrap replications. The phylogentic relationship between the 23 Utah fungal isolates and other related fungi is shown in Figure 1. The ITS plus LSU-based phylogenetic tree revealed two well-supported genera, *Fusarium* and *Alternaria*, with bootstrap values of 99. Within *Fusarium*, four Utah fungal

isolates (1 to 4) were in the phylogenetic clade of *Fusarium brachygibbosum* and six Utah fungal isolates (5 to 10) were in the clade of *Fusarium solani* with a bootstrap value of 89 and 99, respectively. Utah fungal isolate 11 was clustered closely to *Fusarium oxysporum* f. sp. *rapae* and this was supported by a posterior greater than 0.7. Within *Alternaria*, eleven Utah fungal isolates were more closely related to each other than to isolate number 17 and belong to the phylogenetic clade of *Alternaria alternata, Alternaria tenuissima, Alternaria longipes* and *Alternaria mali.*

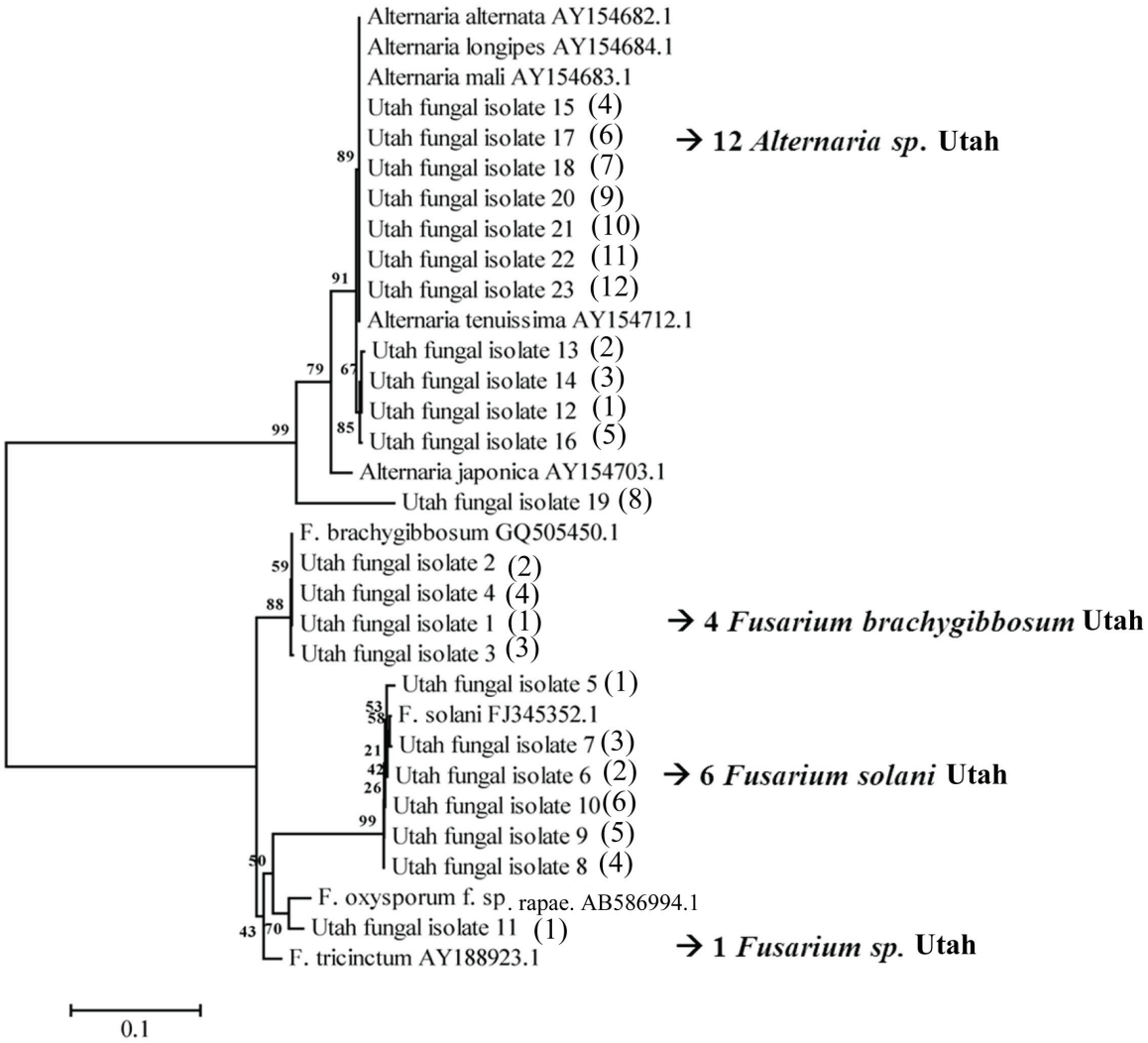


Figure 2. Cluster analysis of Utah fungal isolates constructed with MEGA5 software by the neighbor-joining method based on sequences of ITS region plus LSU (aligned with Clustal W algorithm, MegAlign). The numbers on the nodes indicate bootstrap scores in NJ analyses (1000 steps). The numbers in brackets are the re-assigned isolate numbers after identification of the different Utah fungal isolates to genus/species level. The number on the scale indicates distance level with relative units.

5.2 Metabolic profiling of Utah fungal isolates

The difference of metabolite profiles between the Utah fungal isolates which were identified as *Fusarium solani*, *Fusarium brachygibbosum* and *Alternaria* species by ITS plus LSU sequencing was assessed by liquid chromatography-time-of-flight-mass spectrometry (LC-ToF-MS) analysis. Negatively charged metabolites were selected using the electrospray ionization (ESI) interface in the negative ion mode and they were eluted from the column between 3 and 481s and have mass-to-charge (*m/z*) values ranging from 107 to 1200. Using the conditions mentioned in materials and methods, after data processing, a total of 559 ions were identified (see Supplemental Data 2 for all ions of *Alternaria* sp.).

The cluster analysis of mass profiles resulted in a dendrogram (Figure 3) and showed that the two fungal genera *Fusarium* and *Alternaria* could be seperated. Within the genus *Fusarium, Fusarium solani* and *Fusarium brachygibbosum* cluster separately. The dendrogram shows three major subgroups of *Alternaria*. The first group contains *Alternaria sp*. Utah 4, 8, 10, the second one holds *Alternaria sp.* Utah 1, 11, 12 and the third one has *Alternaria sp*. Utah 9, 2, 3, 5, 6.

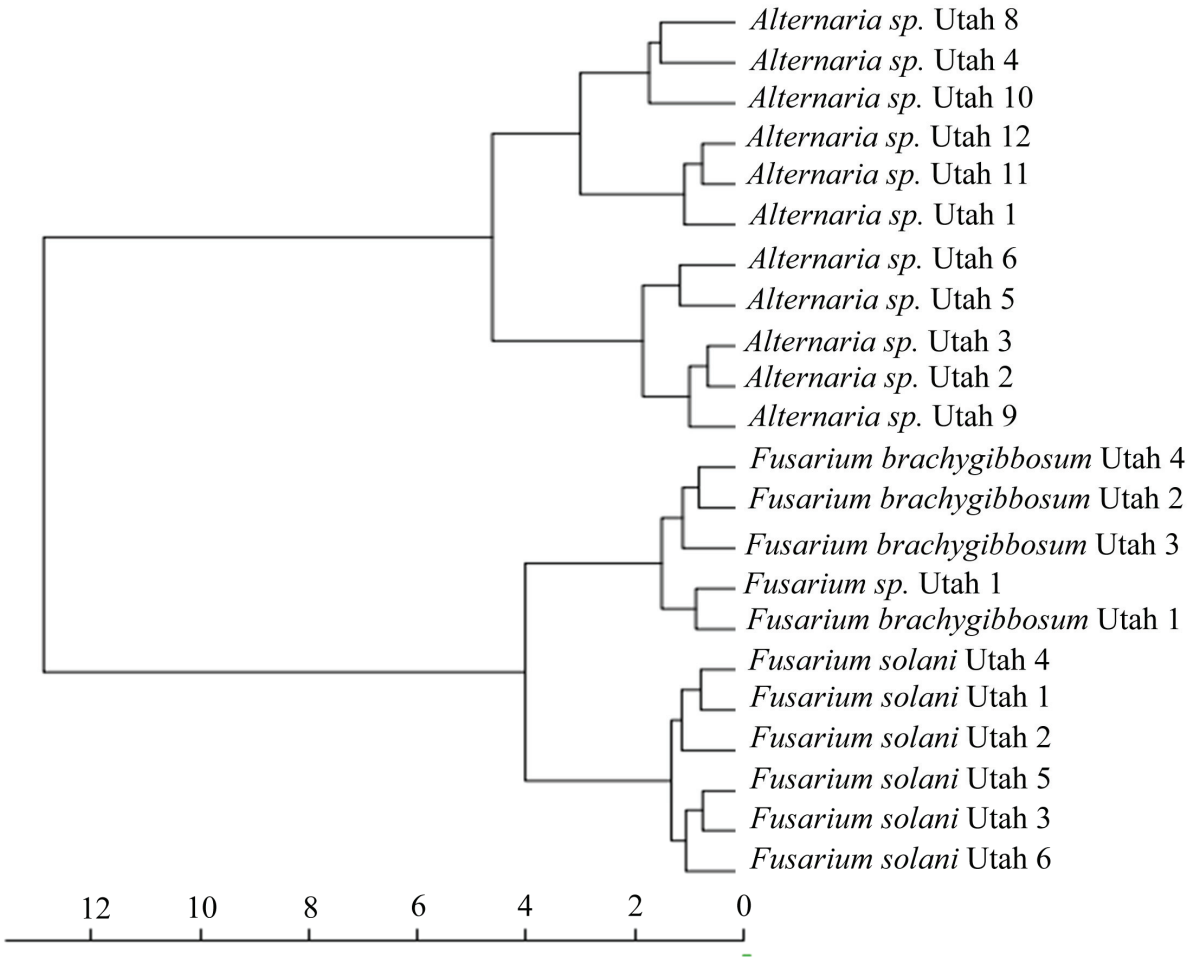


Figure 3: Dendrogram of Utah *Fusarium* and *Alternaria* species group generated by hierarchical clustering in MetaboAnalyst using Ward's linkage clustering method based on metabolic spectral data resulting from LC-ToF-MS using negative ion mode. The numbers on the scale indicate distance level with relative units.

To facilitate the graphical interpretation of the differences among Utah fungal isolates, the data set corresponding to the differentially accumulating ions was analyzed by principal component analysis (PCA). The result is shown in Figure 4. The first and second principal components (PCs) together explained 47.1% of the variation within this data set, and PC1 and PC2 clearly separated the Utah fungal isolates into two different groups, *Fusarium* and *Alternaria.* Metabolites produced by different *Alternaria spp*. seem to differ a lot between isolates, while the metabolite profiles of *Fusarium solani* and *Fusarium brachygibbosum* were much more homgenous.

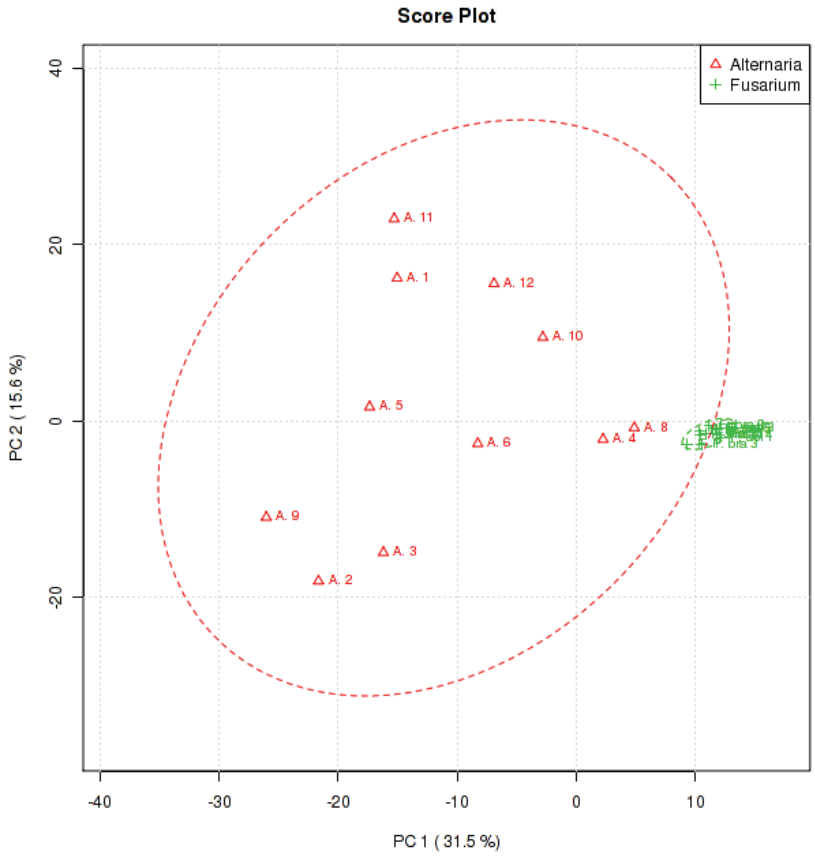


Figure 4. PCA analysis of LC-ToF-MS results comparing *Fusarium* and *Alternaria* isolates. PC1 and PC2 explained together 47.1% of the variance of the samples (the explained variances per component are shown in parentheses). Ellipses delimit the 95% statistical confidence areas for each biological group in the score plots.

A supervised method, partial least squares discriminant analysis (PLSDA) was used to identify ions with stronger impact on the separation of *Alternaria sp*. isolates. To do so, the *Alternaria sp.* isolates were grouped into group 1, 2 and 3 following the dendrogram groups from Figure 3. The PLSDA analysis result is shown in Figure 5. The first and second PCs of the PLSDA analysis explained 40% of the variation in the data set, and these two PCs clearly separated the group 1 of *Alternaria sp.* Utah 4, 8 and 10 from two other groups. *Alternaria sp.* Utah 5 and 6 were clustered into group 3 (95% statistical confidence) together with *Alternaria sp.* Utah 2, 3 and 9.

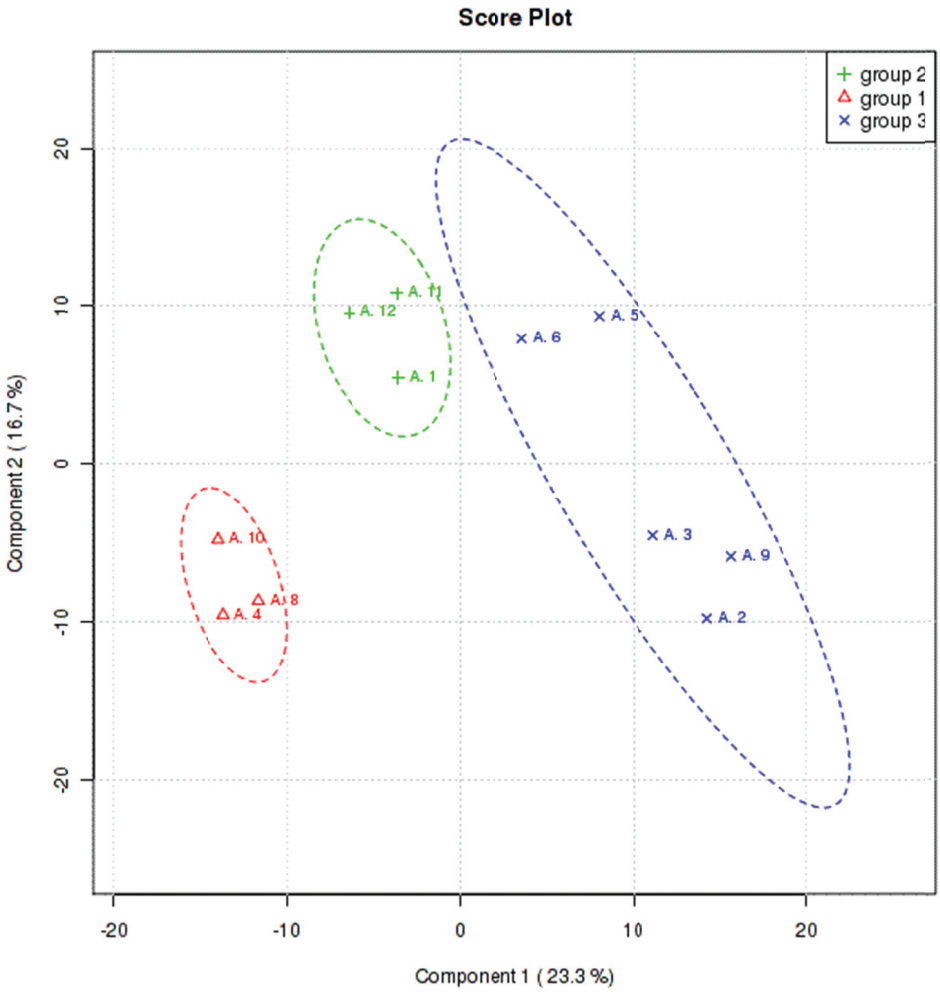


Figure 5: PLSDA analysis of LC-ToF-MS results comparing the three groups of *Alternaria* isolates. PLSDA class separation based on the top two components of PC1 and 2 which explained together 40% of the variance of the samples (the explained variances per component are shown in parentheses). Ellipses delimit the 95% statistical confidence areas for each group in the score plots.

The PLSDA analysis was validated by a permutation test as described in materials and methods and used to calculate the variable importance in the projection (VIP) value to estimate and rank the influence of individual ions on the separation of the samples by the PLSDA model. The higher the VIP value, the stronger its influence on the separation of the samples. A total of 207 ions presented VIP values larger or equal to 1.0 (Supplemental Table 4). A ranking of top 15 ions presenting VIP score values $\geq$ 1.9 is presented in Figure 6. To define the identity of this top 15 ions, a search in the public metabolite database and in custom databases (Nielsen and Smedsgaard, 2003) was performed using the *m/z* values (with an error of ± 0.02) and the retention times. However, none of them was identified.

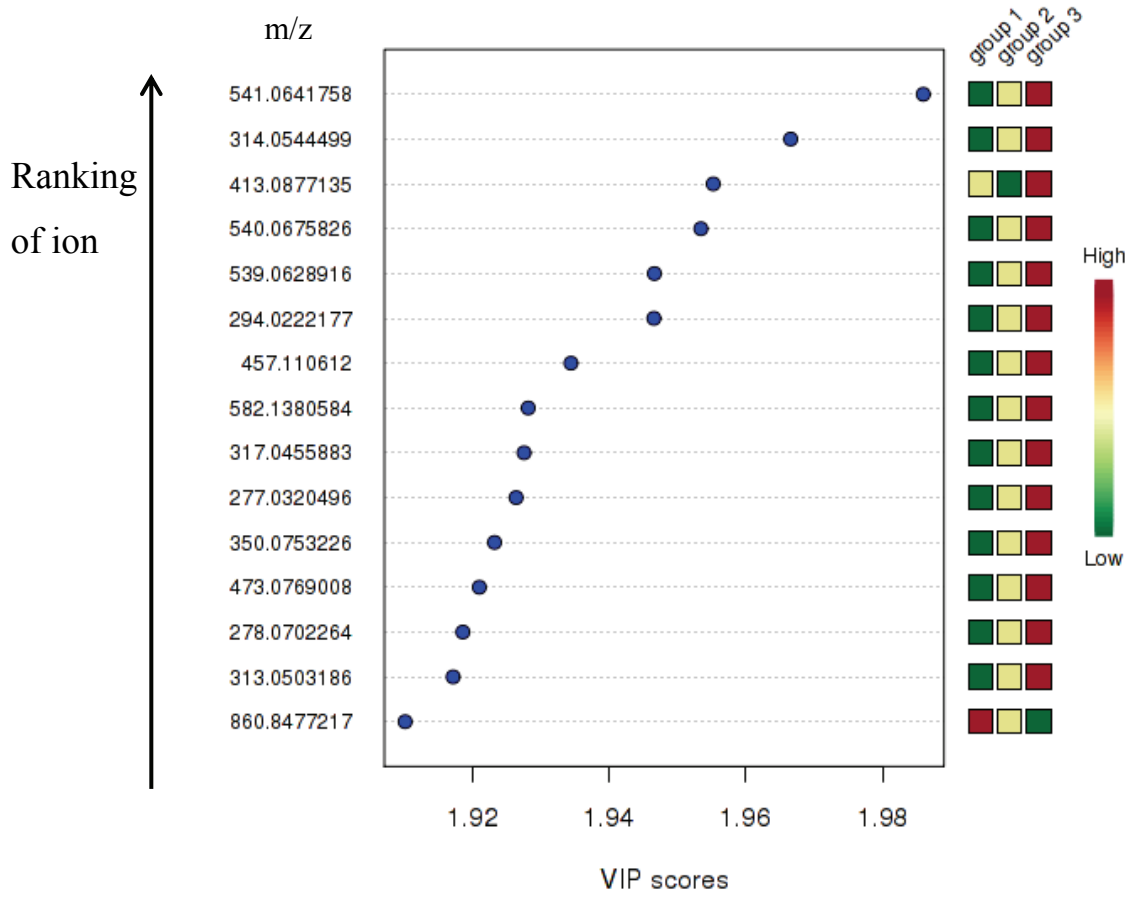


Figure 6: A ranking of top 15 ions of Alternaria presenting VIP score values > 1.0 in the separation of samples by PLSDA. The larger the VIP score, the bigger the ranking value and the stronger the influence of the ion. Three group of *Alternaria sp.* were separated following the dendrogram of metabolic profiling: Group 1 include *Alternaria sp*. Utah 4, 8 and 10; the group 2 contain *Alternaria sp.* Utah 1, 11 and 12; the group 3 contains *Alternaria sp*. Utah 9, 2, 3, 5 and 6. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study.

In summary, by comparison of metabolite profiles between Utah fungal isolates, *F. brachygibbosum* and *F. solani* isolates are separated from each other and from *Alternaria sp.* isolates. In addition, deeper analysis of metabolite profiles revealed justified distinction of the *Alternaria sp.* isolates into 3 groups.

5.3 Classification of *Alternaria sp.* isolates by the presence of known species-specific mycotoxins

Alternaria mycotoxins that have been shown to occur naturally are alternariol (AOH), alternariol monomethyl ether (AME), altertoxin I (AlxI), tentoxin (Ten) and altenuene (Alt) (Weidenborner, 2001). AOH were found to be present in *A. alternata* and *A. tenuissima*, but not in *A.mali* and *A. longipes.* AME was found to be produced by *A. alternata*, *A. tenuissima* and *A. longipes* but not *A. mali*. Ten is produced by *A. alternata* and *A. tenuissima* and AlxI is presented in all four species. The production of important *Alternaria* mycotoxins by *Alternaria* species was summarized in Supplemental Table 5 together with their references.

To detect the presence of those species-specific metabolites, mass and retention time of those compounds accessible at a public database (Nielsen and Smedsgaard, 2003) were used to check the LC-ToF-MS data for the presence of such compounds, allowing for a variation of ± 0.02 for the *m/z* values. The produced *Alternaria* species-specific metabolites found in this analysis are shown in Table 1.

Table 1. Mycotoxins from *Alternaria sp.* Utah isolates assigned by mass over charge values.

Alternaria isolate	Metabolite* (*m/z* value) and peak insensity			
	AOH (259.0592)	AME (273.0752)	AlxI (351.0874321)	Ten (415.2337)
Alternaria sp. Utah 1	275223.8	1417.7	32567.7	143739.7
Alternaria sp. Utah 2	36014.7	1213.6	401.4	41718.4
Alternaria sp. Utah 3	685327.6	1446.7	10401.8	188794.7
Alternaria sp. Utah 4	416540.2	2017.9	3851.7	3979.8
Alternaria sp. Utah 5	32121.9	1227.9	404395.4	16496.7
Alternaria sp. Utah 6	37545.0	1756.5	342584.3	12643.5
Alternaria sp. Utah 8	1057.3	2808.8	13295.6	0.889
Alternaria sp. Utah 9	102667.5	2035.5	48820.2	2483.3
Alternaria sp. Utah 10	125329.2	1854.1	64100.2	22212.1
Alternaria sp. Utah 11	782.4	0.889	197.6	202.7
Alternaria sp. Utah 12	89926.6	434.0	194645.8	13992.6

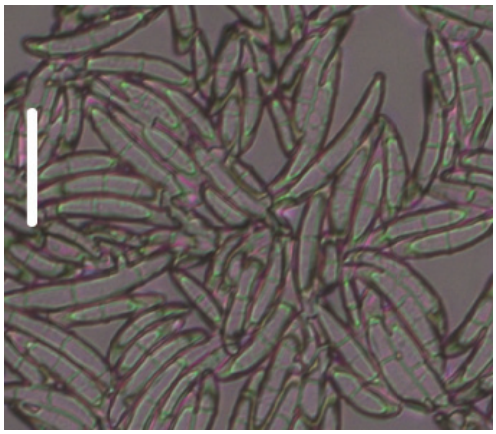
*AOH, alternariol; AME, alternariol monomethyl ether; Alx I, Altertoxin I; Ten, Tentoxin; TeA, tenuazonic acid. "+" presence, "-" absence.

AOH, AME and AlxI and Ten were found to be produced by all Utah *Alternaria* sp. isolates excepted for *Alternaria sp.* Utah 11 and 8 where AME and Ten were almost not detectable. Based on our knowledge about *Alternaria*-specific metabolites (Supplemental Table 5), the presence of all those metabolites indicates that all Utah *Alternaria sp.* isolates belong either to *A. alternata* or *A. tenuissima.* This result indicated that the presence of *Alternaria*-specific mycotoxins could narrow down the possibility of *Alternaria* species assignments for the Utah isolates. However, it was also not sufficient to exactly classify the *Alternaria* isolates up to species level.

5.4 Morphological characterization of Utah fungal isolates by microscopy

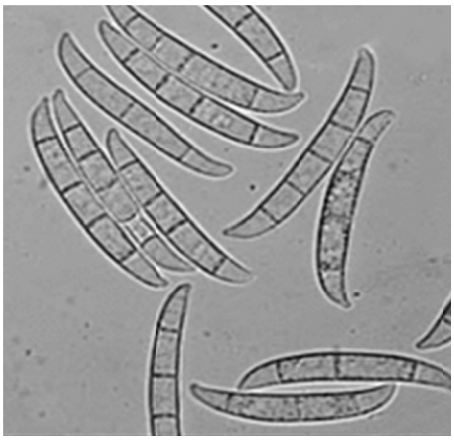
To confirm the identification of *Fusarium solani* isolates resulting from ITS+LSU sequencing and metabolite profiling, Utah fungal isolates were observed under the light microscope after 14 days grown on PDA plates in the dark at room temperature. Typical conidia shape, color, length and septum number were taken into account during observation. Reference conidia pictures of *Fusarium solani* published by David Geiser (Zhang, *et al*., 2008) were used for comparison (Figure 7 B and D). *F. solani* Utah 1 to 4 were confirmed with typical banana-shaped 3- to 5- septate macroconidia (Figure 7 A). Microconidia were abundant and have oval shape with 1- to 2-celled (Figure 7 C).

A



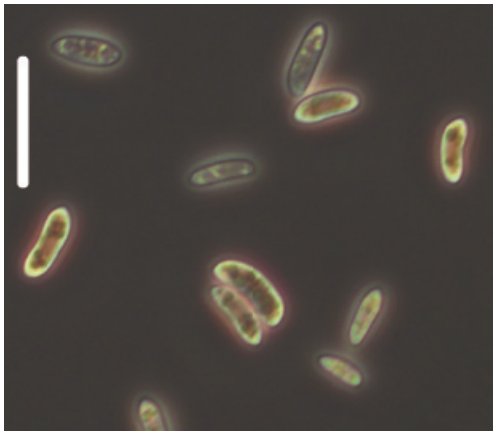
Fusarium solani Utah 2(bar: 20µm)

B



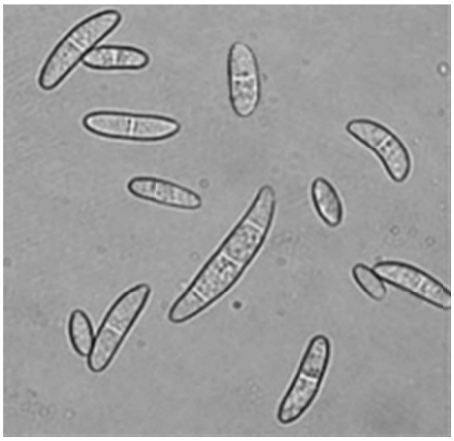
Fusarium solani. Macroconidia. Photo by David Geiser.

C



Furasium solani Utah 4 (bar: 20µm)

D



Fusarium solani. Microconidia. Photo taken by David Geiser

Figure 7. Confirmation of *Fusarium solani* Utah by microscopy. Fungal conidia were observed under microscopy after 14 days growing on PDA plates in the dark at room temperature. **A** and **C**. Conidia of *F. solani* Utah 2 and *F. solani* Utah 4. **B** and **D**. Reference macroconidia and microconidia of *F. solani* observed by David Geiser. White bar presents 20 µm length.

In contrast to *Fusarium solani*, very little information has been published about *F. brachygibbosum*, especially regarding its morphological characteristics. Spore structure of *F. brachygibbosum* Utah 1 to 4 was observed under the microscope where they showed colorless spores, 1-to 2- celled with ellipsoid shape, 4 to 13 µm in length and 2-4 µm in width (Figure 8).

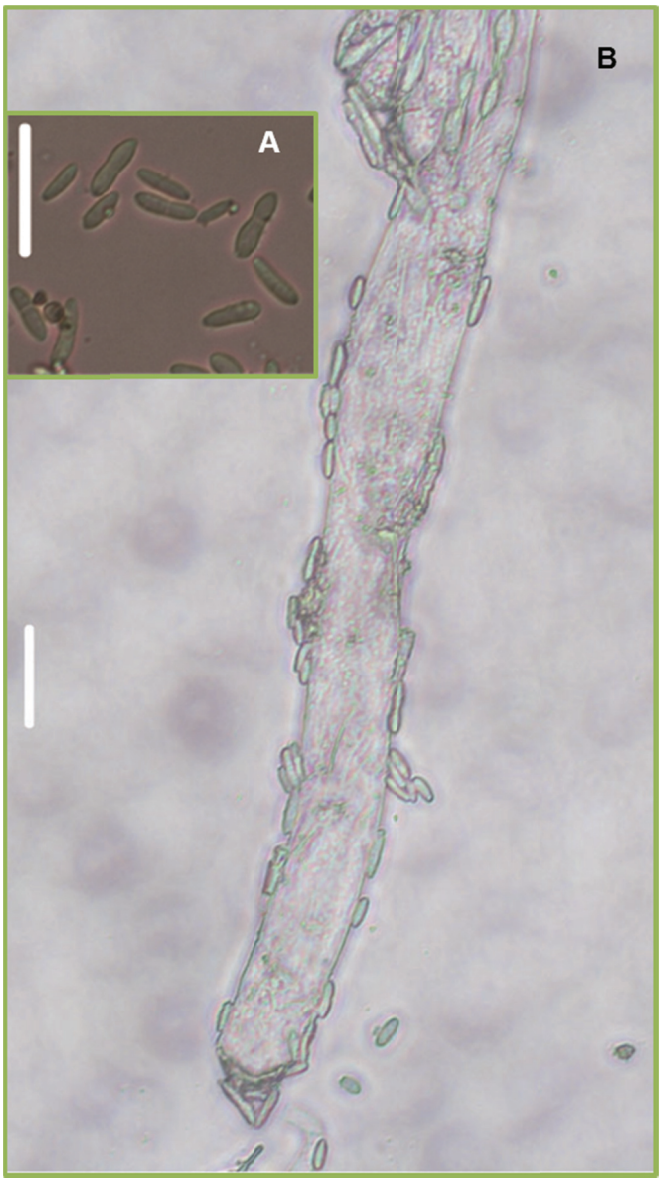


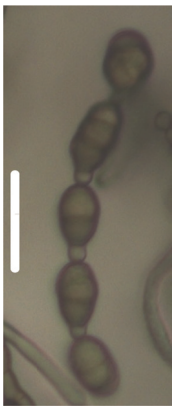
Figure 8. Microscopic characterization of *F. brachygibbosum* Utah 4. A. Fungal spores observed under the microscope after 14 days growing on PDA in the dark at room temperature. **B**. Fungal spore attached on a root hair of *Nicotiana attenuata* (10 days-old seedling). The white bars represent 20 µm length.

All *Alternaria sp.* Utah isolates had conidia shapes typical for *Alternaria*: mostly ovoid with short conical beak at the tip (or beakless). They were multicellular with several vertical and transverse septa (Figure 9 A). The color of conidia was pale brown. Interestingly, *Alternaria sp.* Utah 10 showed typical sporulation of *A. alternata* with a single suberect conidiophore and an apical cluster of conidia chains (Figure 9 C). Branching chains of small conidia separated by short secondary conidiophores are typical for *A. alternata* (Figure 9 B and C). By comparing to reference *A. alternata* conidia pictures published by de Hoog *et al.* (2000), *Alternaria sp.* Utah 10 was recognized as *Alternaria alternata* Utah 10. Because PLSDA analysis revealed that *Alternaria sp.* Utah 4, 8 and 10 were grouped as one thereby these isolates could be identified as *Alternaria alternata* Utah 4, 8 and 10.

A

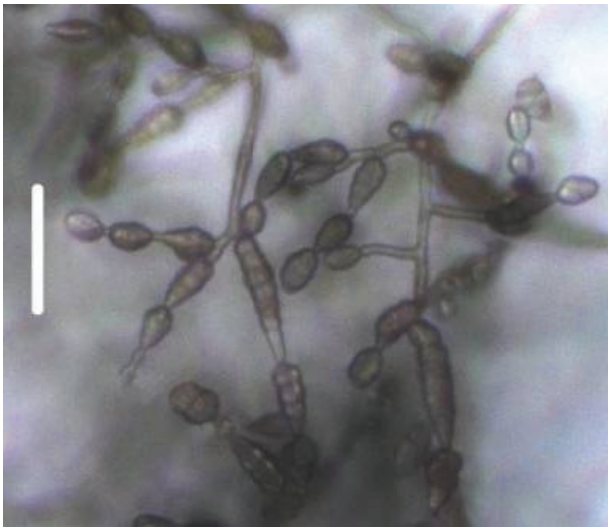
Alternaria sp. Utah 10. Conidia (bar: 20µm)

B



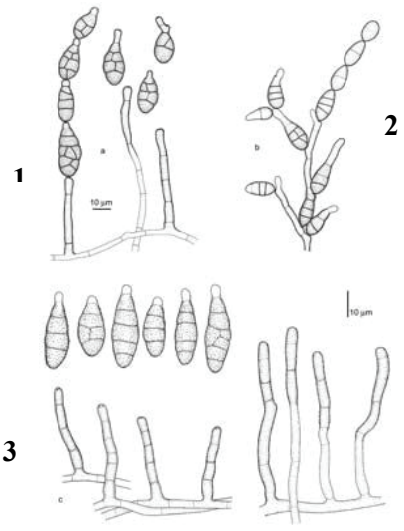
Alternaria sp. Utah 10. Conidia chain (bar: 20µm)

C



Alternaria sp. Utah 10. Sporulation clump and conidia (bar: 20µm)

D



Alternaria alternata, CBS 603.78. Pictures by S. de Hoog

Figure 9. Microscopy of *Alternaria sp.* Utah 10. **A**. *Alternaria*-typical conidia. **B**. *Alternaria*-typical conidia chain. **C**. *S*hort, branching conidia chains. **D**. *Alternaria alternata* sporulation clump and conida, published by de Hoog (1. Conidiophores, liberated conidia and a part of a conidial chain: 2. degenerate subculture on DRYES agar; 3. mature conidiophores and liberated conidia)

In summary, by using different identification approaches including DNA sequencing, microscopy and metabolic profiling, the Utah fungal isolates could be assigned to three *Fusarium* species (*F. solani, F. brachygibbosum* and a not further described *Fusarium sp.*) and to a group of small-spored *Alternaria* comprising *A. alternata* (*A. alternata* Utah 4, 8 and 10), *A. tenuissima*, *A. mali* and *A. longipes*. Successful isolation of those fungi from *N. attenuata* diseased leaves harvested from Utah led to the question of which fungus was actually responsible for the observed disease symptoms. In order to answer this question, it was hypothesized that at least one of the fungal isolates from infected Utah leaves is the causal disease agent. To falsify this, an infection assay of Utah fungi for *N. attenuata* plants was needed to be developed in order to observe the plant´s response to the fungi and checked whether the plants get similar disease symptoms as those in Utah. Therefore, an initial assay was setup to screen for (1) the most suitable isolate from each of the three main species (*F. brachygibbosum*, *F. solani* and *A. alternata*), (2) the optimal plant age for infection and (3) the right infection procedure. To evaluate pathogenicity of all 23 Utah fungal isolates, a detached leaf assay was used.

5.5 Detached leaf assay with Utah fungal isolates on *N. attenuata* leaves

Disease symptom severity of 4 *F. brachygibbosum* Utah isolates, 6 *Fusarium solani* Utah isolates, 12 *Alternaria sp.* Utah isolates and 1 *Fusarium sp*. Utah isolate were tested in a detached leaf assay. The fungal agar plugs (14 d on PDA) were placed on detached leaves of *N. attenuata* and incubated at high humidity. Detached leaves incubated with pure PDA plugs were used as control. The assay was carried out with 12 biological replicates per isolate

After 3 days of incubation, *F. brachygibbosum* Utah 1, 2, 3 and 4 were the first isolates causing visible symptoms (hypersensitive lesions). A hypersensitive reaction is a special sort of necrosis, indicating that the plant cells could recognize fungus and respond to it by undergoing cell death at the infection site in order to prevent further pathogen spread (in case of biotrophic pathogens) (Pontier *et al*., 1998; Kliebenstein and Rowe, 2008). The lesions expanded rapidly from the inoculation point and only two days later started to overlap with each other. To give an example, hypersensitive lesions caused by *F. brachygibbosum* Utah 4 are shown in Figure 10 A. The diameter of hypersensitive lesion after 5 days of inoculation was used to evaluate differences in pathogenicity among *F. brachygibbosum* isolates (Figure 10 B). *F. brachygibbosum* Utah 4 was the most aggressive isolate and caused hypersensitive lesion diameters of 3.03 ± 0.96 cm which was significantly higher than others (one-way ANOVA with LSD posthoc test, $P \leq 0.001$). Followed by less aggressive isolates

which were *F. brachygibbosum* Utah 1 with 2.0 ± 0.7 cm and *F. brachygibbosum* Utah 3 with 2.1 ± 0.4 cm. *F. brachygibbosum* Utah 2 was a "moderate" isolate with a lesion diameter of "only" about 1.8 ± 0.3cm.

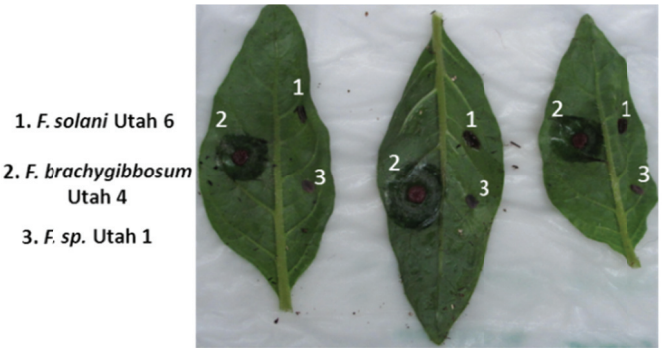
N. attenuata detached leaves inoculated with all *Alternaria sp*. isolates started to show typical symptoms of *Alternaria* infection including chlorosis and necrosis after 5 days of incubation (except for *Alternaria sp.* Utah 10 and 11 which showed chlorotic lesion development already 3 days after inoculation). Chlorosis spread in bright yellow ringshape around the inoculation sites which is typically caused by tentoxin produced by several species of the genus *Alternaria* (Lax *et al*., 1988) (Figure 10 C). Necrotic and chlorotic lesion diameters measured 7 days after inoculation were used to evaluate aggressiveness of the 12 *Alternaria sp*. isolates (Figure 10 D and E). *Alternaria sp.* Utah 10 which caused the biggest necrotic lesion (one-way ANOVA with LSD posthoc test, $P \leq 0.02$) and a large chlorotic lesion diameter (2.1 ± 0.7 cm) appeared to be the most aggressive isolate, followed by *Alternaria sp.* Utah isolates No. 7, 9 and 11. *Alternaria sp*. Utah 3 was rather a moderate isolate while the other isolates (*Alternaria sp.* Utah 1, 2, 4, 5, 6 and 12) were only slightly different to the control.

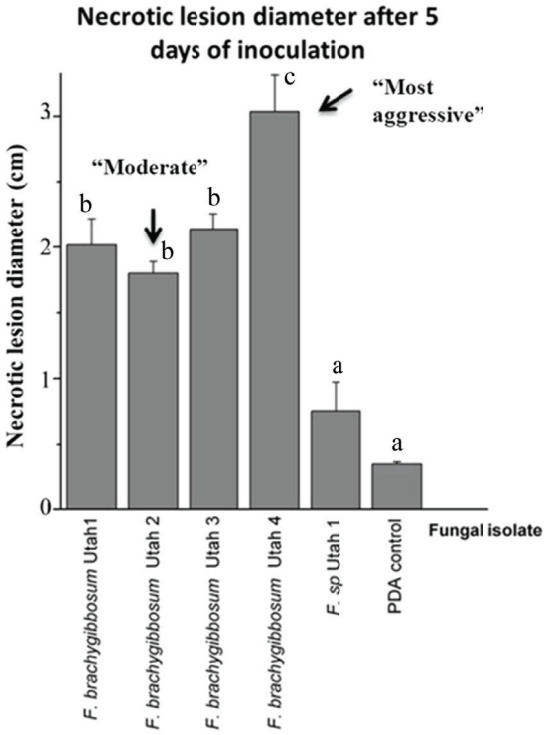
Similar to *Alternaria sp*., *Fusarium solani* inoculated leaves developed chlorosis and necrosis after 4 to 5 days of inoculation. The pathogenicity of different *Fusarium solani* Utah isolates was evaluated based on average values of necrotic and chlorotic lesion diameters (Figure 10 F and G). *F. solani* Utah 6 was shown to be the most aggressive isolate since it caused the biggest necrotic lesion (one-way ANOVA with LSD posthoc test, $P < 0.0001$) and also big chlorotic lesion diameter. *F. solani* Utah 1, 2 and 4 were considered rather moderate isolates since they caused chlorotic lesion diameters comparable to *F. solani* Utah 6 (however did not cause intense necrotic lesions). *F. solani* Utah 3 and 5 did not show differences to control treatment.

In summary, 23 different Utah fungal isolates caused different disease symptoms like necrosis and chlorosis. The level of necrosis and chlorosis were strongly correlated among *Alternaria sp*. isolates but only slightly in *Fusarium solani* isolates. No chlorotic lesion on detached leaves was observed in *F. brachygibbosum* after inoculation. These differences in aggressiveness were useful to identify suitable isolates for another infection assay using intact plants instead of detached leaves and spore suspensions instead of agar plugs containing actively growing fungal culture. Therefore the most aggressive and a rather moderate isolate representing both extremes of the pathogenicity range were chosen to perform a screening assay to setup optimal experimental conditions.

B

A. Necrosis after 3 days of inoculation





C. Necrosis and Chlorosis after 7 days of inoculation

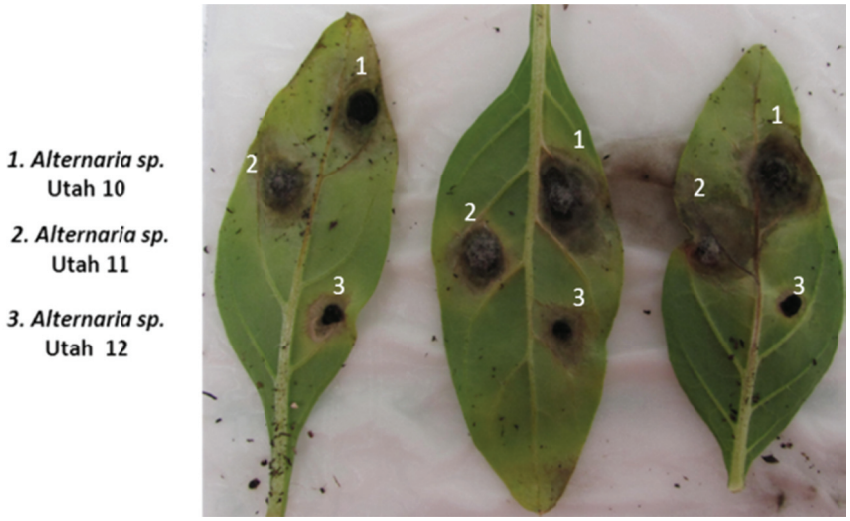


Figure 10. Necrosic and chlorotic lesion diameters on *Nicotiana attenuata* detached leaves after inoculation with Utah fungal isolates. **A**. Necrotic lesions caused by *Fusarisum brachygibbosum* Utah on inoculated leaves 3 days after inoculaton in comparison to other *Fusarium* isolates. **B**. Necrotic lesion diameter of *F. bravhygibbosum* Utah-inoculated leaves 5 days after inoculation. **C**. Necrositic and chlorotic lesions after 7 days of inoculaton with *Alternaria sp.* Utah isolates

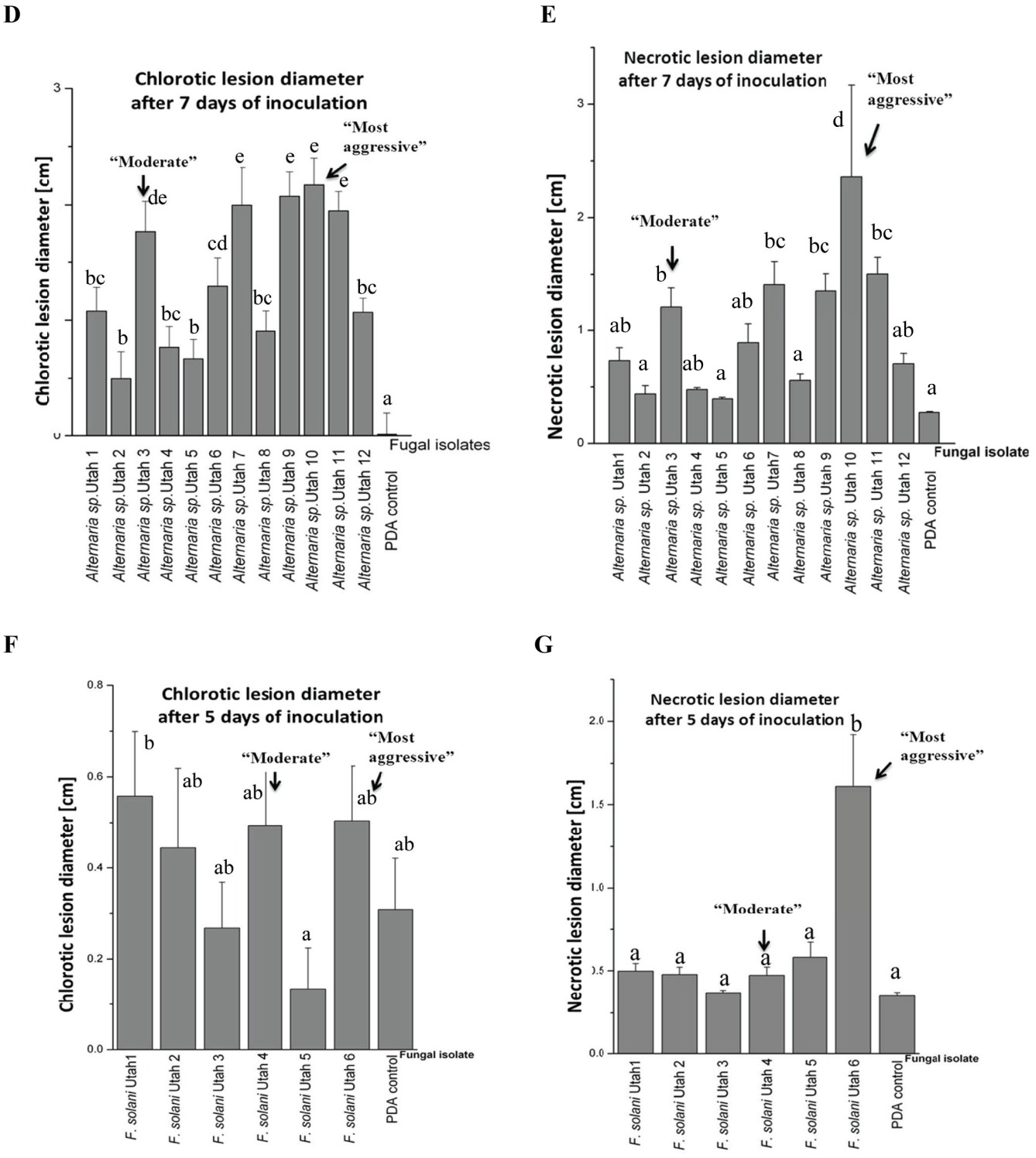


Figure 10 continued. Chlorotic and necrotic lesion diameter on leaves inoculated by *Alternaria sp.* Utah 7 days after inoculation (D and E) or *F. solani* Utah 5 days after inoculation (F and G). Different letters (a-d) indicate significant differences among inoculation of fungal isolates in comparison with PDA control determined by one-way ANOVA with LSD posthoc test ($P \leq 0.05$).

5.6 Method development for an infection assay using spore suspension from Utah pathogenic fungi and intact *N. attenuata* plants

In order to find a suitable setup for infection assays using the Utah fungal pathogens and intact *N. attenuata* plants which is able to discriminate between rather resistant and susceptible genotypes of *N. attenuata*, a low-replicate screening assay was done for each fungal species. The most aggressive and a rather moderate isolate of *F. brachygibbosum* Utah, *F. solani* Utah and *Alternaria sp.* Utah were screened for their pathogenicity at different spore concentrations (10^5, 10^6 and 10^7 spores per milliliter). Since both JA biosynthesis and signal perception by COI1 were known to be important for *F. oxysporum* to affect Arabidopsis plant (Thatcher *et al*., 2009), two transgenic *N. attenuata* lines (ircoi1 and aslox3) were included in this initial assay in order to find suitable experimental designs which allow to distinguish the differences between resistant and susceptible *N. attenuata* genotypes. *N. attenuata* wild type and transgenic plants at different plant age (10 and 20 days-old) were used for the infection. Two different infection methods were tested: root-dip and spraying. Control plants were dipped into or sprayed with 10 mM $MgSO_4$ solution. Development of disease symptoms on infected plants was observed and recorded by estimating the percentage of chlorosis and necrosis. The screening was done for 20 days after the infection. To give an example, the screening result for *F. solani* Utah is shown in Figure 11 and described in detail below.

Infection of only 10 days-old seedlings by spraying of spores from the most aggressive isolate (*F. solani* Utah 6) did not result in any infected plant even with the highest tested spore concentration (10^7 spores/ml) (Figure 11 A). This indicated that spraying was not an appropriate inoculation method for *F. solani* Utah isolates. In contrast, with root-dip method, plants got heavily infected even at the lowest tested spore concentration (10^5spores/ml) (Figure 11 B), indicating that root-dip method was more effective for spore application than spraying. However, 10 days-old plants are very small and as a consequence their biomass available for further analyses (e.g. phytohormone and secondary metabolite quantification) is small. In addition, all 10 days-old seedlings got strong disease symptoms to such an extent that a proper discrimination between susceptible and resistant ones was not possible. Therefore it could be concluded that older plants should be used for the infection assay with *F. solani* Utah 6. Therefore, 20 days-old plants were tested, however still resulted in heavy infection when the aggressive isolate No. 6 was used. In contrast, using the moderate *F. solani* Utah 4 isolate successfully revealed phenotypic differences between genotypes (Figure 11 C, D and 12). To summarize these results, infection with the moderate isolate (*F.*

solani Utah 4) using a spore suspension with the concentration of 10^5 spores/ml for inoculation of 20 days-old *N. attenuata* plants by root-dip method was to be a suitable experimental procedure for *F. solani* Utah to persue the other objective of this master thesis. Similar conclusions were also made for the other isolates, resulting in optimal infection conditions for *Alternaria sp.* Utah 10 (15 days-old plant, 10^5 spores /ml, root-dip inoculation, Supplemental Figure 2 A) and *Fusarium brachygibbosum* Utah 4 (10 days-old plant, 10^7 spores/ml, root-dip inoculation, Supplemental Figure 2 B).

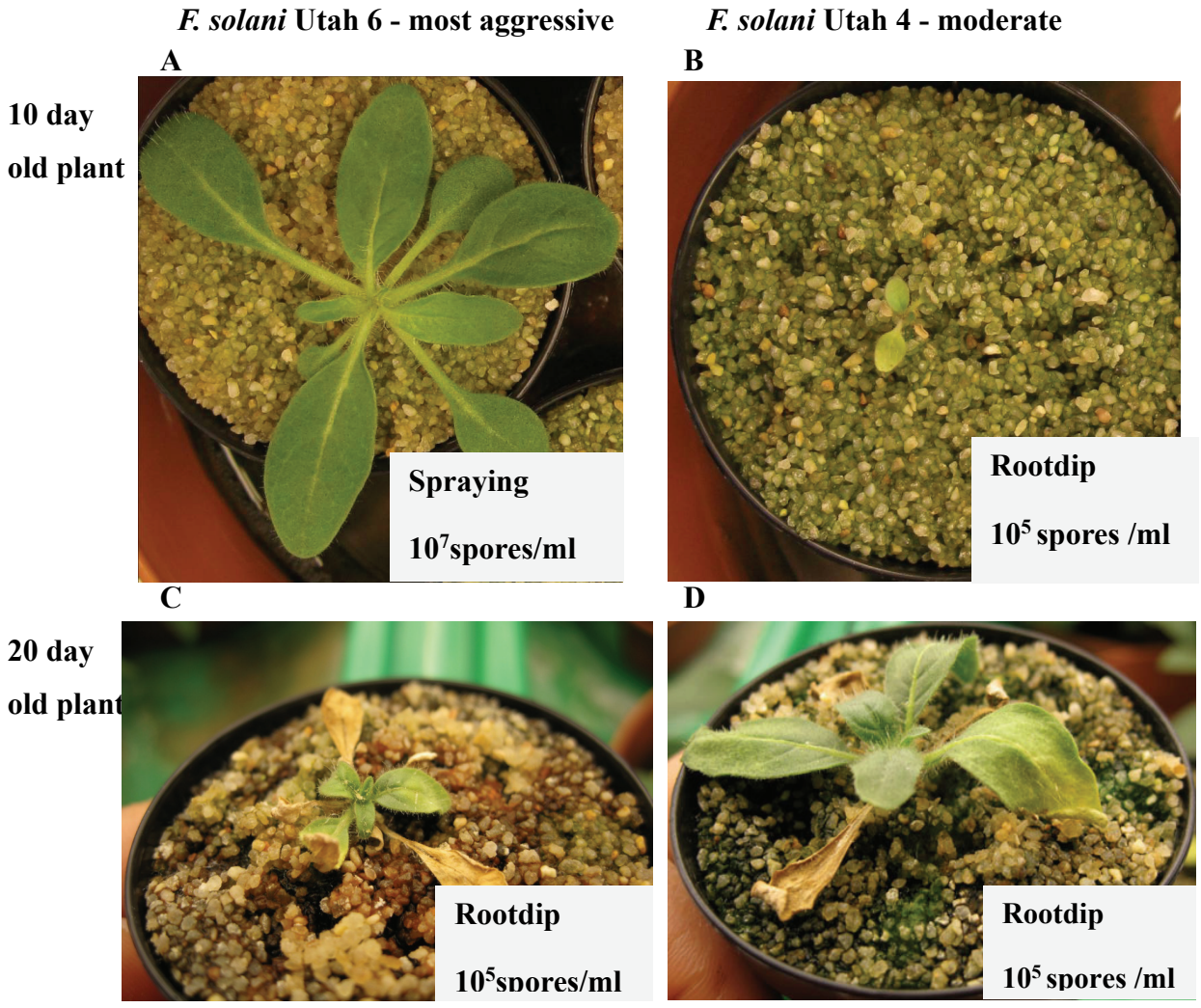


Figure 11. **Disease symptoms from *N. attenuata* plants infected with *F. solani* Utah. A.** Infection of *F. solani* Utah 6 - the most aggressive isolate of *Fusarium solani* Utah to a 10 days-old wild type plant using spraying method with a concentration of 10^7 spores/ml. **B**. Infection of *F. solani* Utah 4 - a rather moderate isolate of *Fusarium solani* Utah to a 10 days-old wild type plant using root-dip method with a concentration of 10^5 spores/ml. **C**. Infection of *F. solani* Utah 6 of a 20 days- old wild type plant using root- dip method with a spore concentration of 10^5 spores/ml. **D**. Infection of *F. solani* Utah 4 at a 20 days-old wild type plant using root-dip method with a concentration of 10^5 spores/ml. The pictures were taken 15 days after infection.

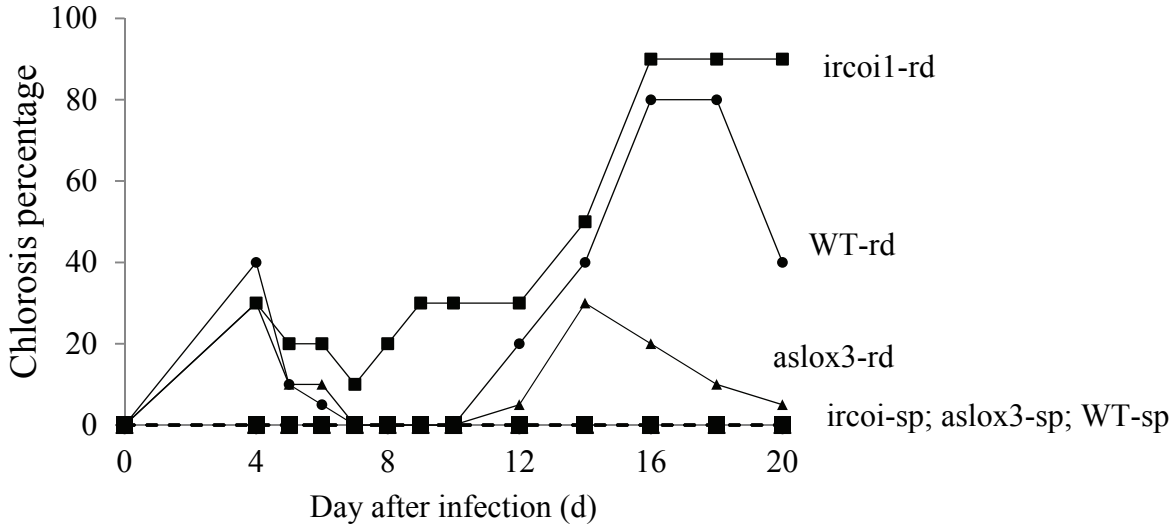


Figure 12. Chlorosis from *N. attenuata* plants infected with *F. solani* Utah 4. Percentage of chlorosis from 20 days-old *N. attenuata* WT plant (circles), aslox3 plant (triangles) and ircoi1plant (squares) which were infected with *F. solani* Utah 4 at a concentration of 10^5 spores/ml by root-dip (rd-solid red line) or spraying (sp-dashed black line) method.

5.7 Infection of *N. attenuata* plants with the three Utah fungal pathogen species individually

The suitable infection setup for each fungal species was used to perform an infection assay in order to find out how *N. attenuata* responds to the pathogens. The assay was done twice and after the first infection assay for each fungus ended, the experimental setup was slightly modified to further optimizie the infection assay. Those minor changes are described in material and methods and in the figure captions. Development of disease symptoms was recorded, plant growth performance (rosette diameter and stalk height) was measure. Plant development characters including time of bolting (appearance of the flower meristem) and time of flowering (first open corolla flower) were recorded and flower morphology was observed during flowering time. Results from two infections are given in Figure 13 for *A. alternata* Utah 10 infection, Figure 14 for *F. brachygibbosum* Utah 4 and Figure 15 for *F. solani* Utah 4

In both infections assays with *A. alternata* Utah 10 disease progressed in a similar way. The first symptoms appeared after 4 to 5 days after infection (dai), typically in form of chlorosis in combination with small and dark brown necrotic lesion spots (Figure 13 A and B). The symptoms continued to increase in severity till 10 dai and some of the infected

plants died during the first infection assay. From 12 to 15 dai, the infected plants started to recover (Figure 13 D and E), especially during the second infection assay where infected plants recovered quickly. Interestingly, in both infection assays, infected plants remained smaller in rosette diameter than non-infected plants till 30 to 35 dai (*t* test, P ≈ 0.002 - 0.04). Consequently, infected plants were delayed in bolting (about 4 days during the first infection assay and 1 day in the second infection assay). The average bolting time is indicated in Figure 13 F-G as downwards pointing arrows (for example 28.9 dai for non-infected (in black) and 33.3 dai for infected plants (in red) during the first infection assay. After 30 dai, all infected plants recovered and reached a similar rosette size as non-infected plants (Figure 13 F and G). During the first infection assay, infected plants got even bigger in rosette diameter than non-infected control plants at the late stage of plant development. Stalk height of non-infected plants was significantly higher than that of *A. alternata* Utah 10-infected plants during the elongation stage (*t* test, P ≈ 0.01 - 0.03) in both infection assays (Figure 13 H and I). Flowering time of infected plants therefore was also delayed by more than 4 days during the first infection assay and almost 2 days in the second infection assay. Surprisingly, at the final stage of plant development (about 60 days-old plant), both infected and non-infected plants reached to the same stalk height. No change in flower morphology was found in infected plants compared to non-infected ones (Figure 13 C).

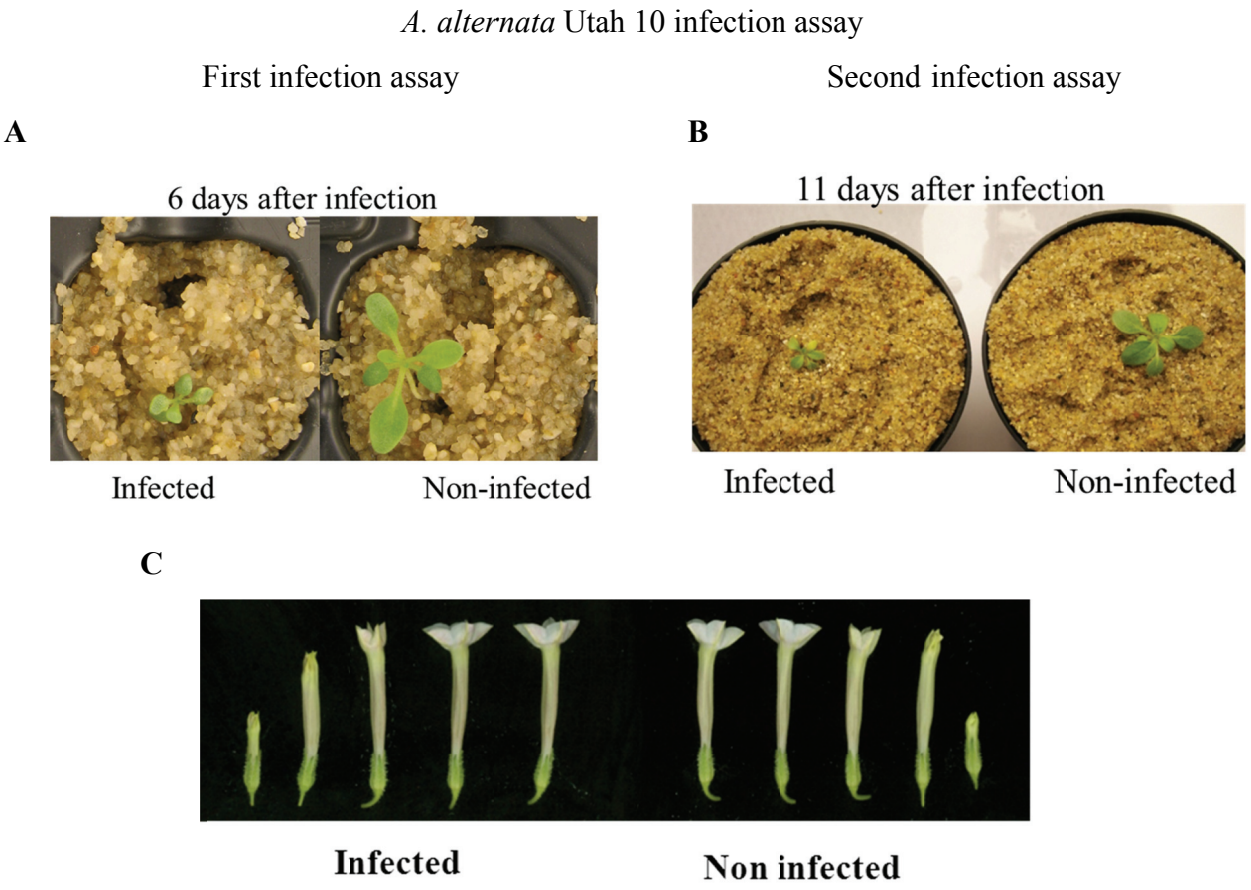


Figure 13. Performance of *Nicotiana attenuata* plants in response to *A. alternata* Utah 10 infection. First infection assay: 10 days-old *N. attenuata* seedlings germinated on GB5 plates were transplanted into Φ3 cm plastic pots and placed in glasshouse (26-28ºC under 16 h supplemental light and 8h dark) for 5 days before the infection. Infection was done by root-dip method with 10^5 spores/ml. After infection, plants were placed in climate chamber (20°C, 16 h light and 8 h dark, 60% humidity). After 5 days when plants were 20 days-old, they were transplanted into Φ10 cm plastic pots. Plants dipped in 10 mM MgSO4 solution were used as controls of non-infected plants. 20 biological replicates were used. **Second infection assay**: 10 days-old *N. attenuata* seedlings germinated from GB5 plates were transplanted into Φ3 cm plastic pots and placed in climate chamber (20°C, 16 h light and 20°C, 8 h dark, 60% humidity) for two days prior to infection. Infection was done by root-dip method with 10^5 spores/ml. At 8 days after infection, 20 days- old plants were transplanted into Φ10 cm plastic pots. Plants dipped in 10 mM MgSO4 solution were used as controls (non-infected plants). 25 biological replicates were used. **A** and **B**. Disease symptoms of *A. alternata* Utah 10 infected plants after 6 days of infection in the first infection assay and 11 dai in the second infection assay. **C.** Flower morphology of *A. alternata* Utah 10-infected plants (right) in comparison to non-infected plants (left).

A. alternata Utah 10 infection assay

First infection assay

Second infection assay

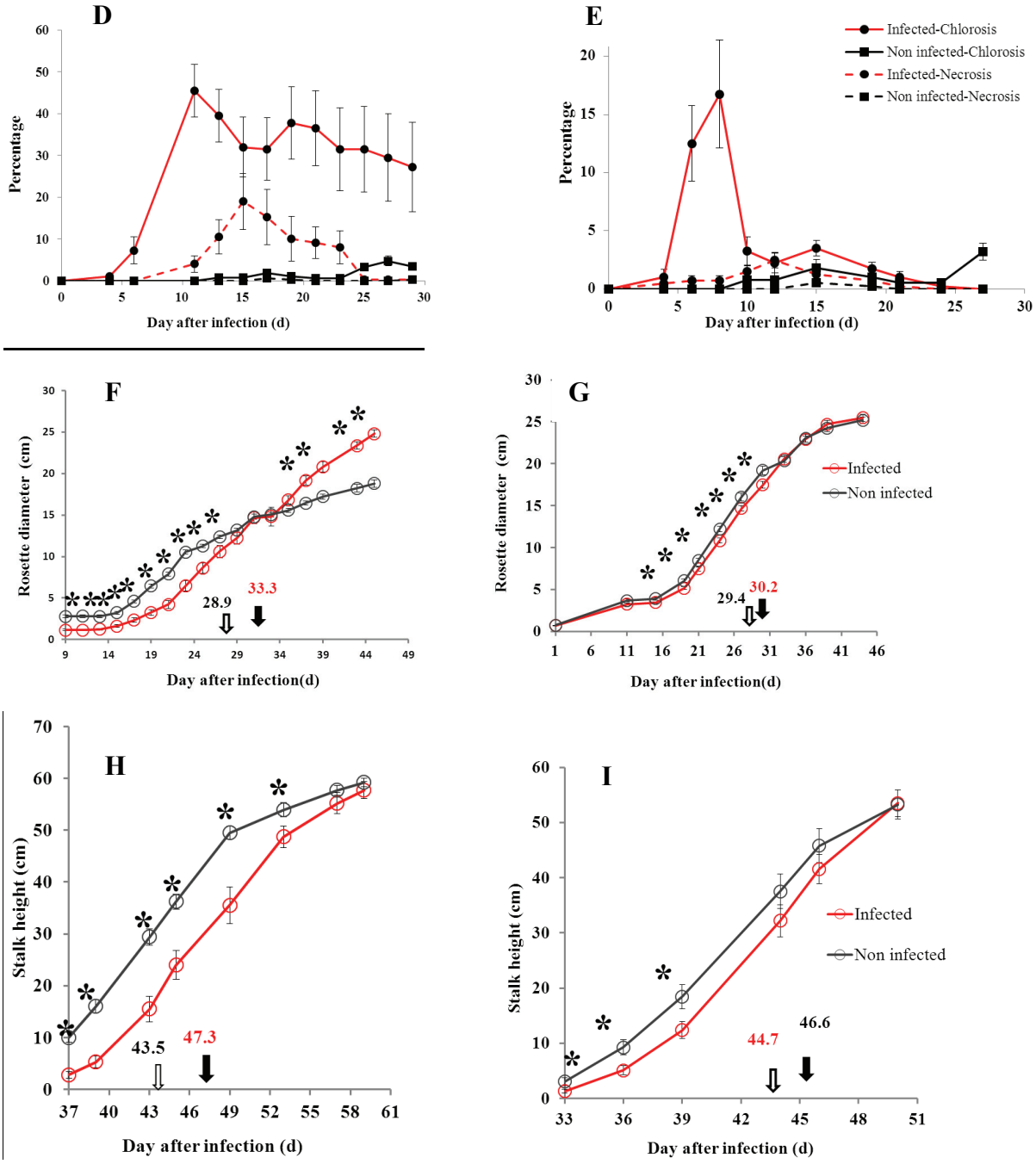


Figure 13 continued. **D** and **E**. Disease symptom development of *A. alternata* Utah 10-infected plants (red) in comparison to non-infected plants (black), presenting the average percentage of chlorosis (solid line), necrosis (dashed line) and wilting (round-dotted line). **F** and **G**. Rosette diameter (mean ± SE) of *A. alternata* Utah 10-infected plants (red line) in comparison to non-infected plants (black line). Numbers on top of the downwards oriented arrows indicate bolting time of infected plants (black arrow) and non-infected plant (white arrow). **H** and **I.** Stalk height (mean ± SE) of *A. alternata* Utah 10-infected plants (black circles) in comparison to non-infected plants (white circles). Numbers on top of the downwards pointing arrows show the average flowering time in infected plants (black arrows) and non-infected plants (white arrows). Asterisks indicate significant differences between infected and non-infected plants (*t* test, $P \leq 0.05$).

The most striking difference between *F. brachygibbosum* Utah 4 and *A. alternata* Utah 10 regarding the observed symptoms is sponaneous wilting occurring in *F. brachygibbosum* Utah 4 infected plants during the second infection assay, beginning after 15-20 days of the infection and lasting until plant reached their final stage. The plants remained green and phenotypically normal (besides reduced rosette and stalk size), but wilted literally overnight. Some infected plants also exhibited stunted growth (Figure 14 B). Others did not show any disease symptoms at the first glance but just had reduced root stability and simply toppled over (Figure 14 C). Chlorosis started after wilting and was followed by necrotic lesions or dried out tissues (Figure 14 F). These results were different from the first infection assay with *F. brachygibbosum* Utah 4 where infected plants did not show wilting symptom but only chlorosis and necrosis (Figure 14 A and E). In addition, in the second infection assay, infected plants showed disease symptoms much later compared to the first infection assay. Moreover, disease symptoms within the 25 replicates varied a lot during the second infection assay. Hypersensitive lesions as observed for the detached leaf assay did not appear when intact plants were used to inoculate with fungal spores.

The two infection assays did not only differ in disease symptom development but also plant growth parameters (rosette diameter and stalk height). In the first infection assay, infected plants were always significantly smaller than non-infected ones in rosette diameter till 40 dai, as well as in stalk height till the final date (*t* test, $P \leq 0.01$) (Figure 14 G and I). Bolting time and flowering time were delayed for approximately 3 and 6 days, respectively. However, once they started to bolt, infected plants grew fast until they reached their final developmental stage, thereby becoming even significantly bigger in rosette diameter compared to non-infected plants (*t* test, $P \leq 0.02$). In contrast, during the second infection assay, *F. brachygibbosum* Utah 4 infected plants grew in a comparable rate to non-infected plants. During the first 30 dai, rosette diameter was similar in both treatment groups until they started to bolt (Figure 14 H). As a result, there was no difference between bolting time of infected and non-infected plants. Despite no significant difference could be detected for stalk height (Figure 14 K) between non-infected and infected plants, rosette diameter of un-infected plants at the late stage was significantly bigger than that in infected plants (*t* test, $P \approx 0.01$ - 0.05) and flowering time was delayed for almost one and a half days. Similar to *A. alternata* Utah 10 infected plants, *F. brachygibbosum* Utah 4-infected ones showed no difference in their flower morphology in comparison to non-infected plants (Figure 14 D) during both infection assays.

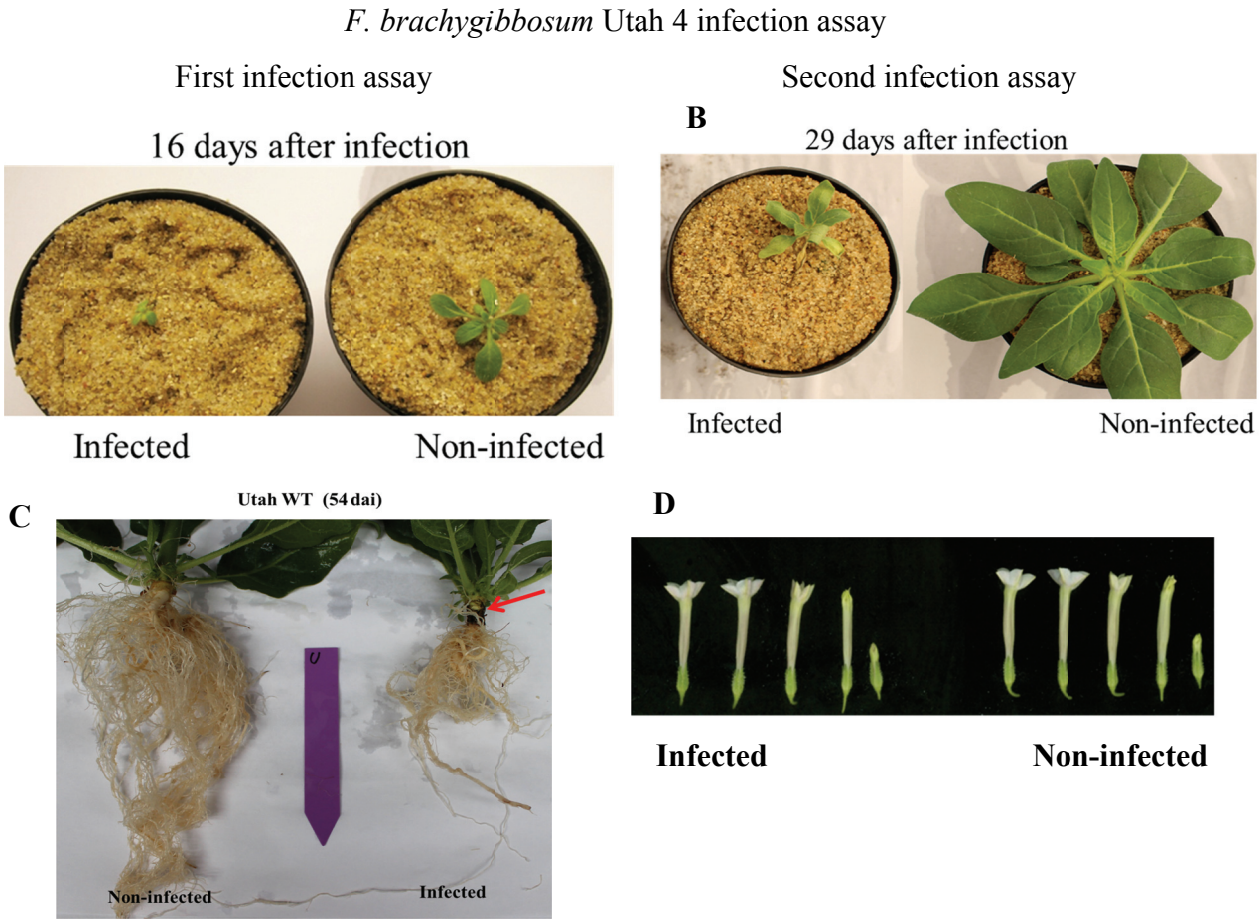


Figure 14. Performance of *Nicotiana attenuata* plants in response to *F. brachygibbosum* Utah 4. First infection assay: 10 days-old *N. attenuata* seedlings germinated on GB5 plates were used directly for infection by dipping seedling roots into a suspension of 10^7 spores/ml. Plants were then grown into Φ3 cm plastic pots and placed in climate chamber (20°C, 16 h light and 20°C, 8 h dark, 60% humidity). After 10 days, when plants were 20 days-old, they were transplanted into Φ10 cm plastic pots. Plants dipped in 10 mM $MgSO_4$ solution were used as controls of non-infected plants. 20 biological replicates were used. **Second infection assay**: 8 days-old *N. attenuata* seedlings germinated on GB5 plates were transplanted into Φ3 cm plastic pots and placed in a climate chamber (20°C, 16 h light and 20°C, 8 h dark, 60% humidity) for two days prior to the infection. Infection was done by root-dip method with 10^7spores/ml. At 10 days after infection, 20 days-old plants were transplanted into Φ10 cm plastic pots. Plants dipped in 10 mM $MgSO_4$ solution were used as controls of non-infected plants. 25 biological replicates were used. **A** and **B**. Disease symptoms of *F. brachygibbosum* Utah 4-infected plants after 16 days of infection in the first infection assay and 29 dai in the second infection assay. **C**. *Fusarium* crown rot symptom of a *F. brachygibbosum* Utah 4-infected plant which did not show symptom of wilting, chlorosis or necrosis and look phenotypically normal. **D**. Flower morphology of *F. brachygibbosum* Utah 4-infected plants (right) in comparison to non-infected plants (left)

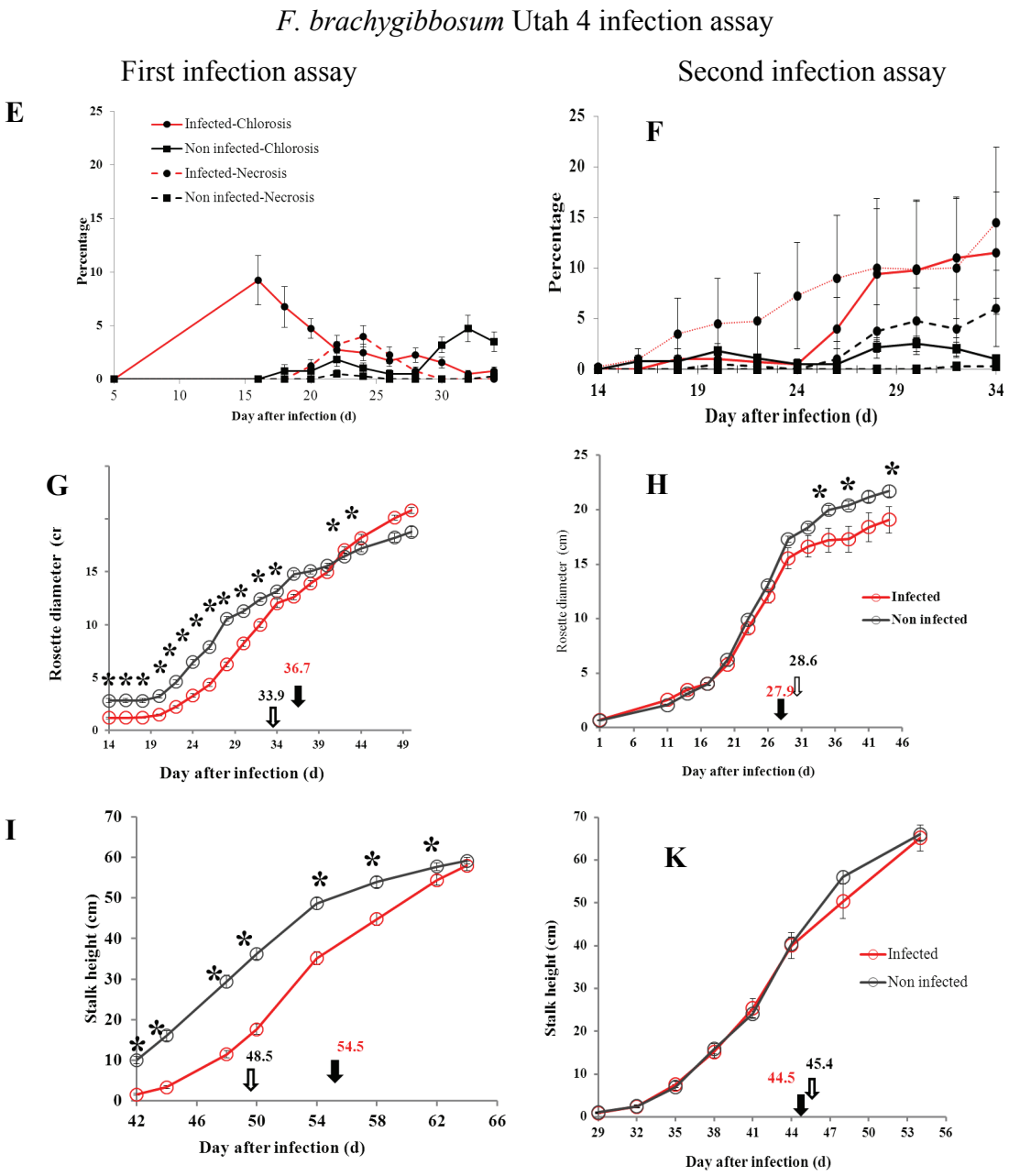


Figure 14 continued. E and **F**. Disease symptoms of *F. brachygibbosum* Utah 4 infected plants (red line) in comparison to non-infected plants (black line), presented the average percentage of chlorosis (solid line), necrosis (dashed line) and wilting (round-dotted line). **G** and **H**. Rosette diameter (mean ± SE) of *F. brachygibbosum* Utah 4 infected plants (red line) in comparison to non-infected plants (black line). Numbers on top of the downwards pointing arrows show the average bolting time in infected plants (red) and non-infected plants (black).**I** and **K.** Stalk height (mean ± SE) of *F. brachygibbosum* Utah 4 infected plants (red line) in comparison to non-infected plants (black line). Numbers on top of the downwards pointing arrows show the average flowering time in infected plants (red) and non-infected plants (black). Asterisks indicate significant difference between infected and non-infected plants (*t* test, $P \leq 0.05$).

The infection assay with *F. solani* Utah 4 was done only once because of risk assessment reasons (classification changed from SI to SII) with 25 biological replicates following the setup regarded as the most suitable one (defined via the initial screening assay mentioned above). Twenty days-old plants were infected with a suspension of 10^5 spores /ml by root-dip method. The plant performance result is shown in Figure 15. Infected plants started to show chlorosis at 4 to 6 dai as the first disease symptom (Figure 15 D). The chlorotic lesions expanded quickly, reaching the maximum percentage of chlorosis at 8 to 10 dai. Necrotic lesions developed after chlorosis, however it was not abundant with less than 10 %. Few heavily diseased plants had curly leaves and shortened, bent flowers with protruding pistils as shown in Figure 15 A and B. The growth at rosette stage was reduced throughout development in infected plants (Figure 15 E), which caused retarded bolting time (1 day delayed). Stalk height was significantly lower in infected plants compared to non-infected plants ((Figure 15 C and F) but flowering time was not differred between infected and uninfected plants (Figure 15 F). Similar to the first infection assays with the two other fungi species (*A. alternata* Utah 10 and *F. brachygibbosum* Utah 4), rosette diameter of infected plants became bigger after bolting than in control plants

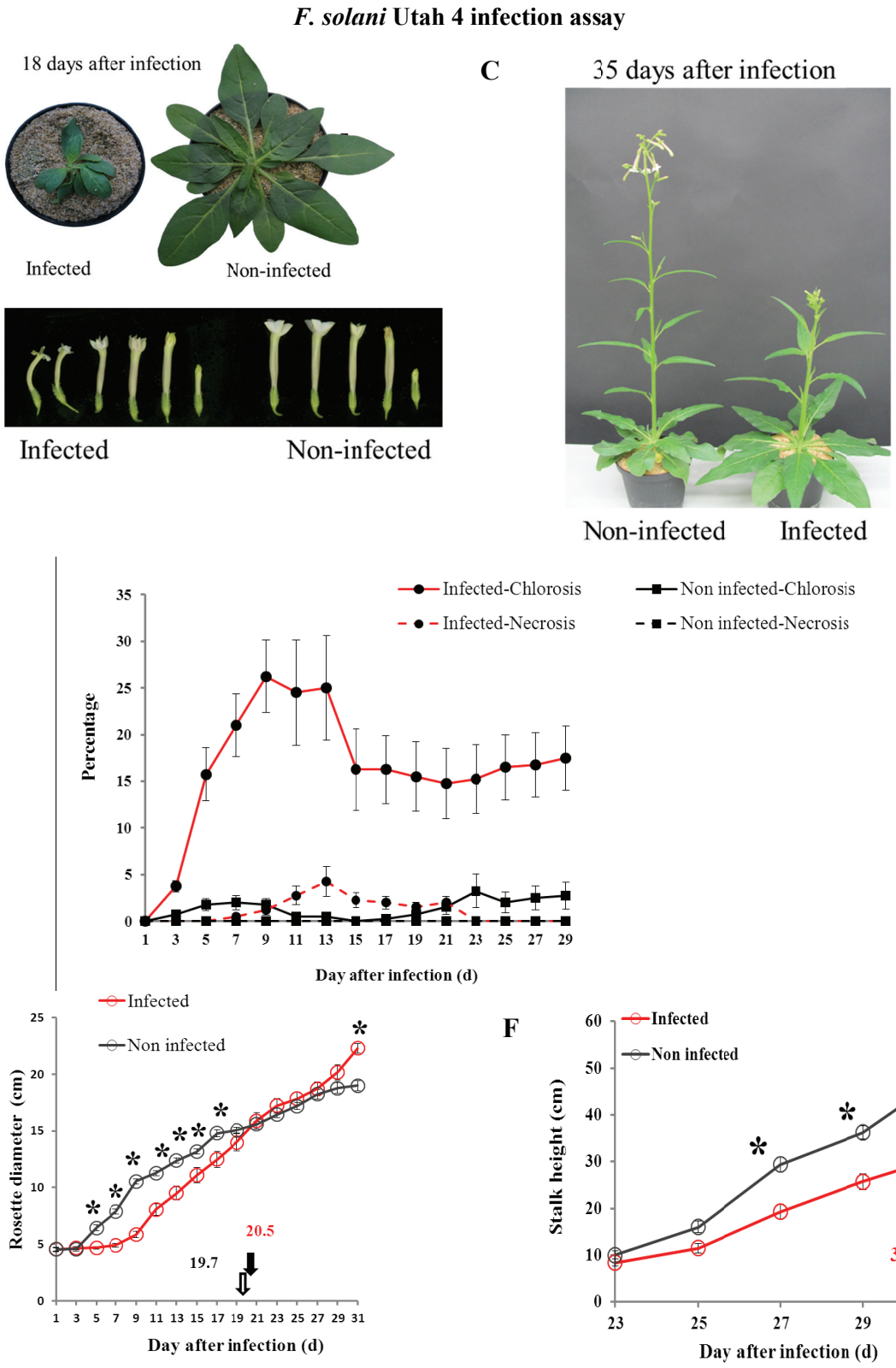


Figure 15. Performance of *Nicotiana attenuata* plant in response to *F. solani* Utah 4. 10 days-old *N. attenuata* seedlings germinated on GB5 plates were transplanted into Φ3 cm plastic pots and placed in glasshouse (26-28ºC under 16 h supplemental light and 8 h dark) for 10 days prior to infection. Infection was done by root-dip method with 10^5 spores/ml. After the infection, plants were grown in Φ10 cm plastic pots in climate chamber (20°C, 16 h light and 20°C, 8 h dark, 60% humidity). Plants dipped in 10 mM $MgSO_4$ solution were used as controls

(non-infected plants). 20 biological replicates were used. **A**. Disease symptoms of *F. solani* Utah 4-infected plants after 18 days of infection. **B**. Comparison of plant morphology at 35 dai between infected right) and non-infected plant (left). **C.** Flower morphology of heavily diseased plants compared to non-infected plants. **D**. Disease symptom development of *F. solani* Utah 4-infected plants (red line) in comparison to non-infected plants (black line), presented as average percentage of chlorosis (solid line) and necrosis (dashed line). **E**. Rosette diameter (mean ± SE) of *F. solani* Utah 4-infected plants (red line) in comparison to non-infected plants (black line). Numbers on top of the downwards pointing arrows show the average bolting time in infected plants (red) and non-infected plants (black). Stalk height Mean ± SE cm of *F. solani* Utah 4 infected plants (red line) in comparison to non-infected plants (black line). Numbers on top of the downwards pointing arrows show the average flowering time in infected plants (red) and non-infected plants (black). Asterisks indicate significant differences between infected and non-infected plants (*t* test, $P \leq 0.05$).

5.8 Changes in phytohormone levels of *N. attenuata* plants infected with the Utah pathogenic fungi

To get a first impression on how the above described phenotypic observations might be connected to changes in phytohormone levels, *A. alternata* Utah 10, *F. brachygibbosum* Utah 4 and *F. solani* Utah 4-infected plants were harvested at three different time points: the first plants were collected at the time point of infection (0d-control), the second batch of plants at 2 days after infection when the infected plants had no disease symptoms and the third sampling time point was chosen according to the time when the first disease symptoms appeared at the infected plants. Detailed information on sampling time points are given in the Figure captions. The method of phytohormone extraction and quantification is described in materials and methods. Phytohormones important for pathogen defense including salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA) were measured. In addition, JA conjugated to isoleucine (JA-Ile) and (9S, 13S)-12-oxo-phytodienoic acid (OPDA) were also quantified. Besides these oxylipins (JA, JA-Ile and OPDA), other groups of oxylipins such as divinylethers and C12-derivatives (C12) resulting from the hydroperoxide lyase (HPL) pathway includes 12-oxo-(9Z)-dodecenoic acid (traumatin), 9-hydroxy-12-oxo-(10E)-dodecenoic acid (OH-traumatin) and (2E)-dodecenedioic acid (traumatic acid) were quantified. Since each fungal infection assay using *F. brachygibbosum* Utah 4 and *A. alternata* Utah 10 was repeated, the phytohormone levels were quantified also twice and it was referred to gram fresh weight in the first and gram dry weight in the second infection assay. Tissue harvesting time points were also differed between assays depending on the disease progression.

N. attenuata plant's response to *A. alternata* Utah 10 infection during the two infection assays is shown in Figure 16. In the first assay, infection by *A. alternata* Utah 10 lead to induction of SA at 2dai (ANOVA, $F_{4;10} = 4.041$, P = 0.021) (Figure 16 A) and JA at 4 dai (ANOVA, $F_{4;10} = 2.477$, P = 0.027) (Figure 16 B). In the second assay, the previously observed induction of SA and JA in infected plants was confirmed by a SA burst at 2 dai (ANOVA, $F_{4;16} = 17.115$, P = 0.004) and 8 dai (ANOVA, $F_{4;16} = 17.115$, P = 0.00004) (Figure 16 J) and JA at 2 dai (ANOVA, $F_{4;16} = 17.466$, P = 0.00002) (Figure 16 K). However, the general pattern of those phytohormones in the two infection assays varied. SA was already decreased at 4 dai in the first infection assay but still increased at 8 dai in the second infection assay. JA was not yet induced at 2 dai in the first assay but already highly induced at the second infection assay. Despite that, the induction of SA and JA after infection by *A. alternata* Utah 10 was clearly confirmed in both infection assays. This hints to a hemibiotrophic lifestyle of *A. alternata* Utah 10 since such microorganisms are known to induce both, SA and JA upon infection (Park *et al*., 2009).

In the second infection assay, as a consequence of JA induction, JA-conjugated isoleucine (JA-Ile) was also increased at 2 dai (ANOVA, $F_{4;19} = 10.243$, P = 0.0004) (Figure 16 M). OPDA which occurs as an intermediate metabolite during JA biosynthesis was also induced at 2 dai with very high amounts of almost 600 nmol per gram dry weight, this concentration was significantly higher than that of non-infected plants (ANOVA, F4;16 = 11.809, P = 0.00007) (Figure 16 N). In addition, ABA was induced at 2 dai (ANOVA, $F_{4;19}$ = 53.768, P < 0.000001) (Figure 16 L), being also a stress indicator. Interestingly, three main C12-derivatives, traumatin, traumatic acid and OH traumatin, produced by the HPL pathway in *N. attenuata* were also induced. The traumatin level in infected plants at 2 dai was almost 5-fold higher than that of non-infected plants (ANOVA, $F_{4;16} = 51.310$, P < 0.00001) (Figure 16 O). Traumatic acid and OH traumatin, which are in their biosynthesis downstream of traumatin (Kallenbach *et al*., 2011), were induced also at 2 dai (ANOVA, $F_{4;18} = 9.232$, P = 0.01) (Figure 16 P) and 8 dai (ANOVA, $F_{4;20} = 18.447$, P = 0.00001) (Figure 16 Q), respectively. Moreover, colneleic acid (9-DVE-18:2) , a product of DES pathway, was significantly increased in infected plants at 2 dai compared to non-infected plants (ANOVA, $F_{4;16} = 11.81$, P = 0.00007) (Figure 16 R). Notably, these inductions were not observed in the first infection assay (Figure 16 C-I).

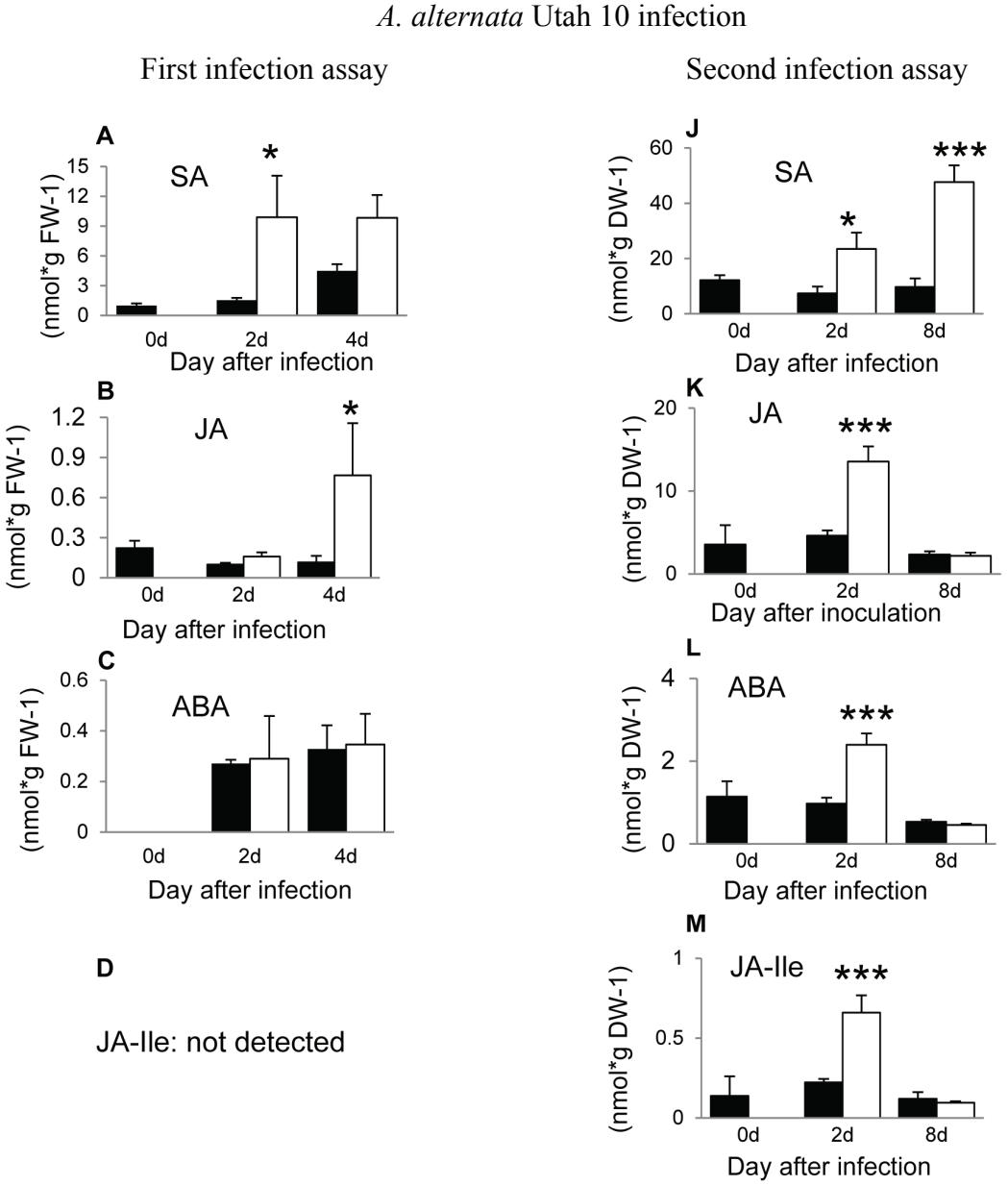


Figure 16. Phytohoromone levels in *Nicotiana attenuata* plants in response to *A. alternata* Utah 10 infection. Infection setup and growth condition for both infection assays are described in detail in the caption of Figure 13. Plants were harvested at three time points, the first time point was at the day of the infection (0d), the second time point was 2dai (2d) and the third time point was 4 dai (4d) in the first and 8 dai (8d) for the second infection assay. Three biological replicates (three pooled seedlings) and five biological replicates (three pooled seedlings) were used for the first and second infection assay at each time point analyzed. Phytohormone concentration was calculated in nmol per gram of fresh weight (nmol*gFW^{-}1) in the first infection assay and in nmol per gram of dry weight (nmol*gDW^{-}1) in the second infection. The name of each phytohormone is indicated in the according figure panel. ABA: abscisic acid, SA: salicylic acid, JA: jasmonic acid, JA-Ile: JA conjugated to isoleucine, OPDA: (9S, 13S)-12-oxo-phytodienoic acid. Black and white bars represent phytohormone levels of non-infected (black) and infected plants (white), respectively. Asterisks indicate significant differences between infected and non-infected plants at a given time point (one-way ANOVA with LSD posthoc test, $P \leq 0.05$).

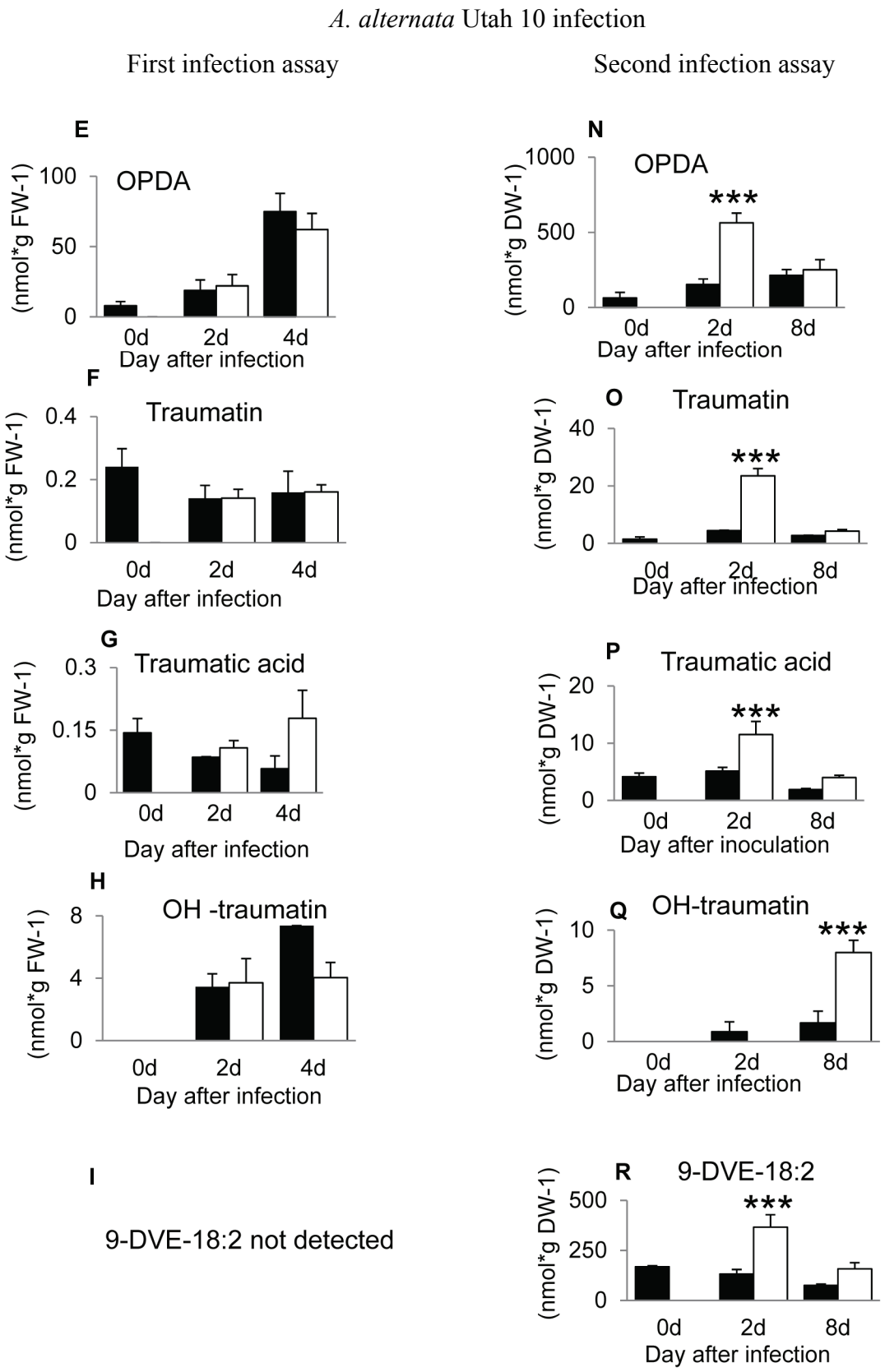


Figure 16 continued. Phytohoromone levels in *Nicotiana attenuata* plants in response to *A. alternata* Utah 10 infection.

In the first infection assay with *F. brachygibbosum* Utah 4, infected plants had higher concentrations of SA and JA compared to non-infected plants (Figure 17 A and B). Especially levels of SA increased dramatically at 2 dai, at least 3-fold compared to the SA concentration of non-infected plants (ANOVA, $F_{4;10} = 44.956$, $P \leq 0.000001$). At 4 dai, SA levels were reduced, but still at a higher level than in non-infected plants (ANOVA, $F_{4;10} = 44.956$, $P = 0.0039$). In addition, JA levels kept increasing also at 4 dai and infected plants had more JA in comparison to control plants (ANOVA, $F_{4;10} = 4.389$, $P = 0.006$). No induction in other measured phytohormones was found in this first infection assay. In the second infection assay, the induction of SA was not significant although this trend is still visible at 2 and 4 dai (ANOVA tested, Figure 17 E). JA could not be detected. However, traumatic acid and 9-OH tramatin were induced at 7 dai (Figure 17 G and H). Especially the concentration of OH traumatin was significantly higher in infected plants compared to non-infected plants (ANOVA, $F_{4;20} = 34.497$, $P = 0.00002$).

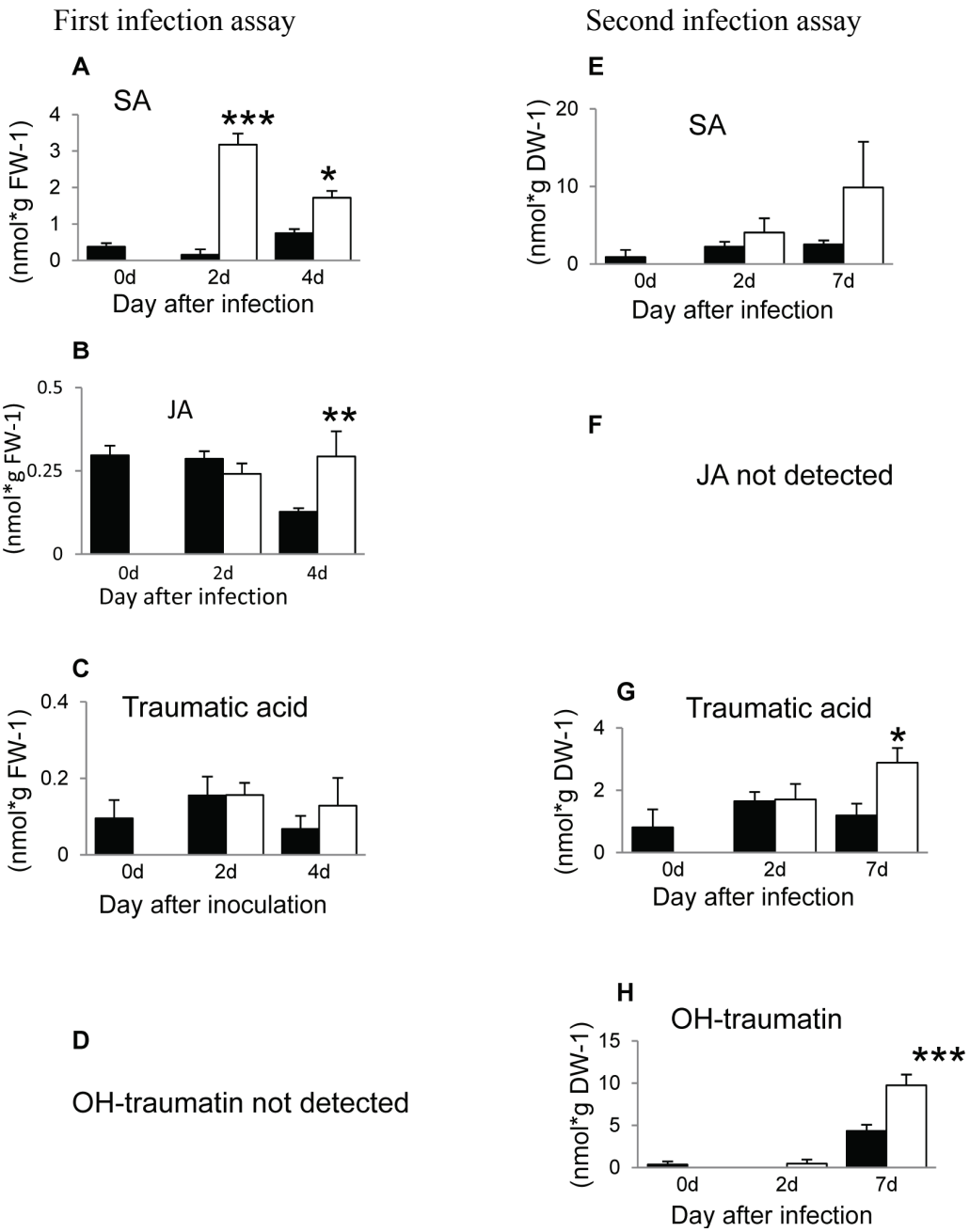


Figure 17. Phytohoromone levels of *Nicotiana attenuata* plants in response to *F. brachygibbosum* Utah 4 infection. Infection setup and growth condition for both infections are described in detail in the caption of Figure 14. Plants were harvested at three time points, the first time point was at the day of the infection (0d), the second time point was 2dai (2d) and the third time point was 4dai (4d) in the first and 7 dai (7d) for the second infection assay. Three biological replicates (three pooled seedlings) and five biological replicates (three pooled seedlings) were used respectively for the first and second infection at each time point. Phytohormone concentration was calculated in nmol per gram of fresh weight (nmol*gFW-1) in the first infection assay and in nmol per gram of dry weight (nmol*gDW-1) in the second infection assay. The name of each phytohormone is indicated in each figure panel. SA: salicylic acid and JA: jasmonic acid. Black and white bars presents phytohormone levels of non-infected (black) and infected plants (white). Asterisks indicate significant differences between infected and non-infected plants at each time point (one-way ANOVA with LSD posthoc test, $P \leq 0.05$.

In case of *F. solani* Utah 4 infection, SA concentration in infected plants was induced at 8 dai (ANOVA, $F_{4;10} = 3.278$, $P = 0.03$) (Figure 18 A). A JA burst could be detected also in infected plants at 3 dai (ANOVA, $F_{4;9} = 6.207$, $P = 0.035$) dai and 8 dai (ANOVA, $F_{4;9} = 6.207$, $P = 0.017$) (Figure 18 B). The induction of both, JA and SA, indicates that *F. solani* Utah 4 could be a hemibiotrophic fungal species.

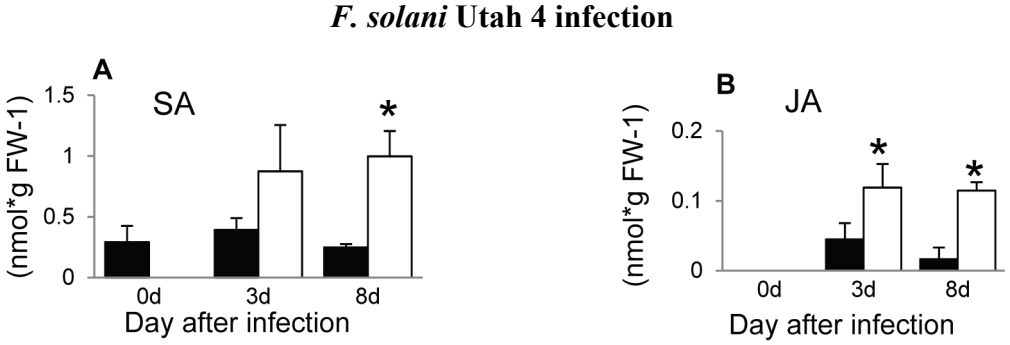


Figure 18. Phytohoromone levels of *Nicotiana attenuata* plants in response to *F. solani* Utah 4 infection. Infection setup and growth condition for both infection assays are described in detail in the caption of Figure 15. Plants were harvested at three time points, the first time point was at the day of the infection (0d), the second time point was 3 dai (3d) and the third time point was 8 dai (8d). Three biological replicates (three pooled seedlings) were used. Phytohormone concentration was calculated in nmol per gram of fresh weight (nmol*gFW^{-}1). The name of each phytohormone is indicated in according figure panel. SA: salicylic acid and JA: jasmonic acid. Black and white bars represent phytohormone levels of non-infected (black) and infected (white) plants. Asterisks indicate significant differences between infected and non-infected plants at a given time point (one-way ANOVA with LSD posthoc test, $P \leq 0.05$).

5.9 Changes in secondary metabolite levels of *N. attenuata* plants infected with the Utah pathogenic fungi

To determine whether induced JA levels in infected *N. attenuata* plants are reflected by changes in JA-inducible secondary metabolites or not, Utah fungi-infected plants were harvested for secondary metabolite extraction. Infection assays with each fungus species (*A. alternata* Utah 10, *F. brachygibbosum* Utah 4 and *F. solani* Utah 4) were performed analogous to those described above. Harvesting times for secondary metabolite extraction were the same as for the phytohormone analysis as previously described. The number of biological replicates was five for each treatment at every sampling time point. The measured

JA-inducible secondary metabolites in *N. attenuata* plants were nicotine, crypto-chlorogenic acid (CCA), caffeoyl putrescine (CP), dicaffeoyl spermidine (DCS) (Keinanen *et al*., 2001; Kessler and Baldwin, 2002; Kaur *et al*., 2010). Besides those, some other secondary metabolites not induced by JA such as rutin and chlorogenic acid (CA) (Keinanen *et al*., 2001; Kessler *et al*., 2004; Wu *et al*., 2008) were also quantified. The result from the first and second infection assay were compared and only secondary metabolites which showed significant changes between infected and uninfected plants in one of the assays are mentioned here.

F. brachygibbosum Utah 4 was able to induce almost all measured secondary metabolites. As shown in Figure 19 A-E at the first infection assay, all six secondary metabolites were induced in infected plants. Nicotine and rutin concentrations in infected plants were significantly higher than that of non-infected plants at 4 dai (nicotine: ANOVA, $F_{4;20} = 30.630$, $P \leq 0.00001$; rutin: ANOVA, $F_{4;20} = 18.681$, $P = 0.00001$). CA, CCA and DCS levels were already increased at 2 dai (CA: ANOVA, $F_{4;20} = 42.121$, $P = 0.01$; CCA: ANOVA, $F_{4;20} = 27.221$, $P = 0.03$; DCS: ANOVA, $F_{4;20} = 23.256$, $P = 0.028$) and increased even further until 4 dai, where the concentrations of CA, CCA and DCS in infected plants were not only significantly higher than those of non-infected plants, but also than those of infected plants at 2 dai (ANOVA, $P < 0.0001$). By contrast, in the second infection assay, where JA levels were not detectable, also no significant difference in all analyzed secondary metabolites except for nicotine was recorded. The level of nicotine was at least 2-times higher in infected plants compared to non-infected plants (ANOVA, $F_{4;17} = 9.913$, $P = 0.008$) (Figure 19 F).

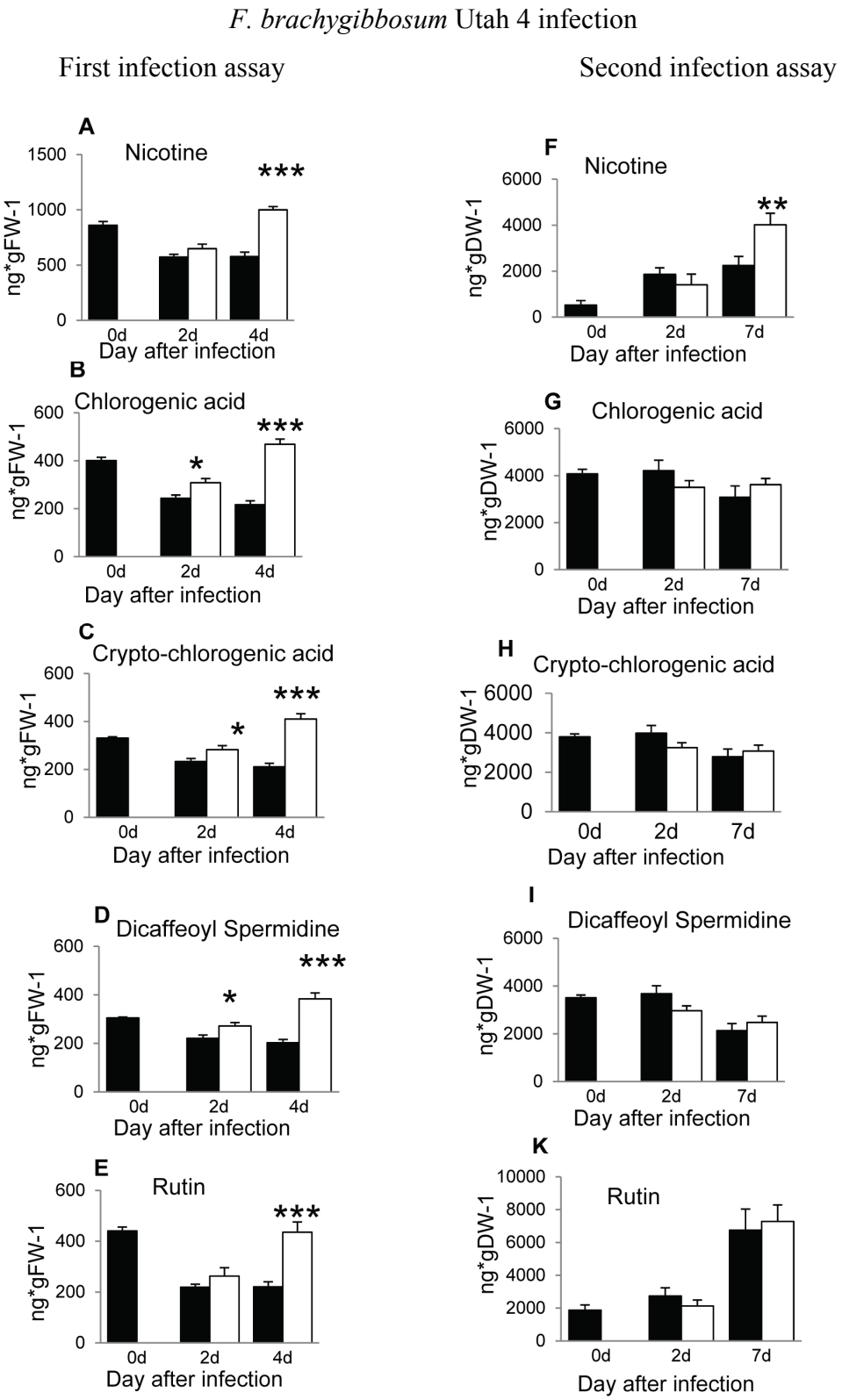


Figure 19. Secondary metabolite levels of *Nicotiana attenuata* plants in response to *F. brachygibbosum* Utah 4 infection. Infection setup and growth conditions for both infections were described in detail in the caption of Figure 14. Plants were harvested at three time points, the first time point was at the day of the infection (0d), the second time point was 2 dai (2d) and the third time point was 4 dai (4d) in the first infection and 7 dai (7d) for the second infection. Five biological replicates

(one seedling per replicate) were used for both infections at each time point for infected and non-infected plants. Secondary metabolite concentration was calculated as nanogram per gram of fresh weight (ng*gFW^{-1}) in the first infection and in nanogram per gram of dry weight (ng*gDW^{-1}) in the second infection. The name of each secondary metabolite is indicated in each figure panel. Black and white bar represent secondary metabolite levels of non-infected (black) and infected (white) plant respectively. Asterisks indicate significant differences between infected and non-infected plants at a given time point (one-way ANOVA with LSD posthoc test, $P \leq 0.05$).

Similar to *F. brachygibbosum* Utah 4 infection, *A. alternata* Utah 10 infected plants also had an induction of CA, CCA and DCS. However, this induction can be seen only in the second infection (Figure 20 D-F) and not in the first infection assay. The concentrations of those three secondary metabolites at 2 dai were remarkably higher in infected plants in comparison to non-infected plants (CA: ANOVA, $F_{4;20} = 75.823$, $P \leq 0.0001$; CCA: ANOVA, $F_{4;20} = 86.983$, $P \leq 0.0001$; DCS: ANOVA, $F_{4;20} = 98.979$, $P \leq 0.0001$). The concentration of CA, CCA and DCS in infected plants was then reduced to a comparable level to that of control plants at 8 dai. Nicotine and rutine were not induced in *N. attenuata* plant after *A. alternata* Utah 10 infection.

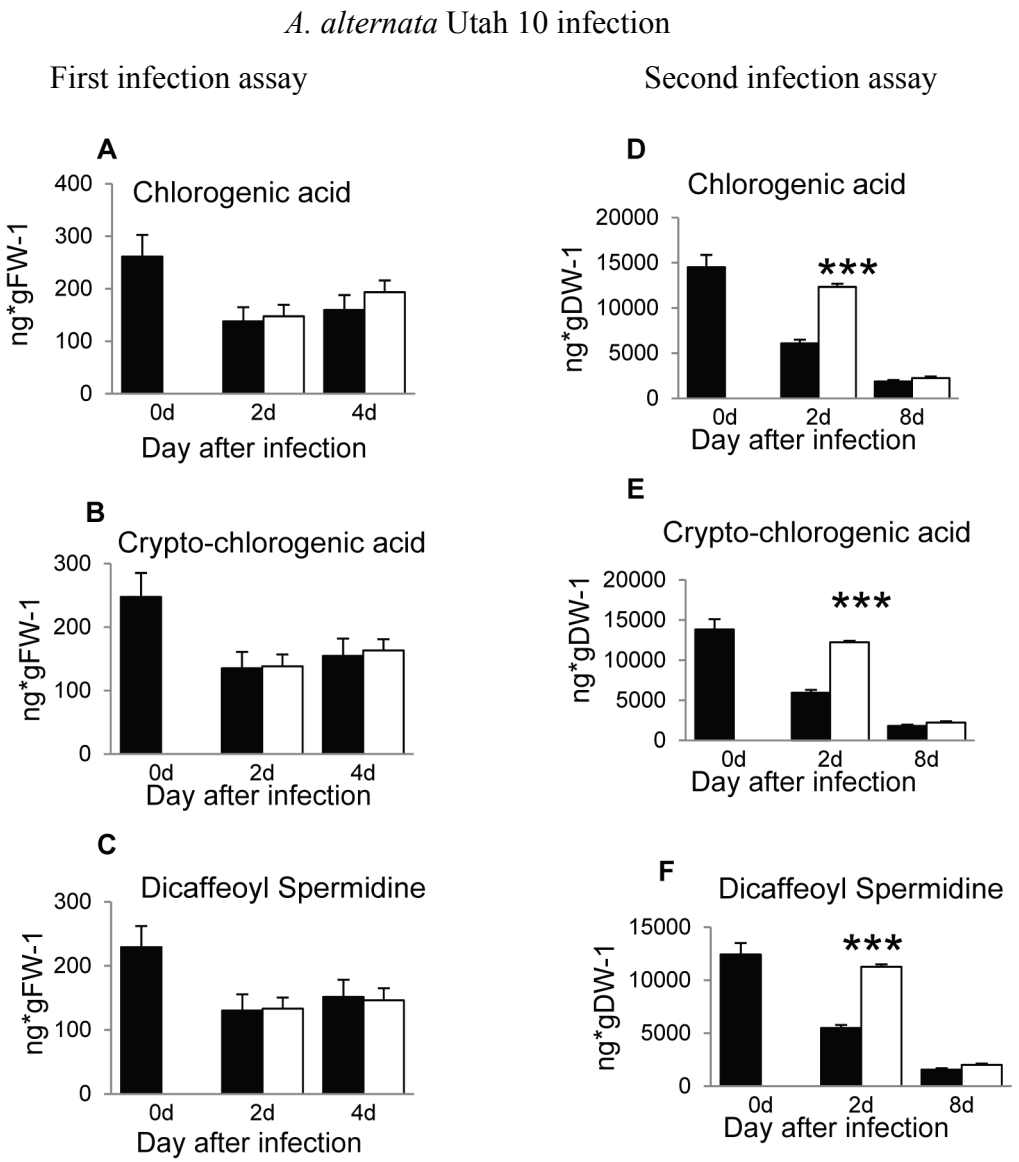


Figure 20. Secondary metabolite levels of *Nicotiana attenuata* plants in response to *A. alternata* Utah 10 infection. Infection setup and growth conditions for both infections were described in detail in the caption of Figure 13. Plants were harvested at three time points, the first time point was at the day of the infection (0d), the second time point was 2 dai (2d) and the third time point was 4 dai (4d) in the first infection and 8 dai (8d) for the second infection. Five biological replicates (one seedling per replicate) were used for both infections at each time point for infected and non-infected plants. Secondary metabolite concentration was calculated as nanogram per gram of fresh weight (ng*gFW^{-}1) in the first infection and in nanogram per gram of dry weight (ng*gDW^{-}1) in the second infection. The name of each secondary metabolite is indicated in each figure panel. Black and white bar represent secondary metabolite levels of non-infected (black) and infected (white) plant respectively. Asterisks indicate significant differences between infected and non-infected plants at a given time point (one-way ANOVA with LSD posthoc test, $P \leq 0.05$).

F. solani Utah 4 infection did not lead to the induction of SA, JA in infected *N. attenuata* plants at 3 dai and 8 dai as well as all tested secondary metabolite, except for chlorogenic acid. The levels of CA in infected plants were considerably higher than that of non-infected plants at 8 dai (ANOVA, $F_{4;20} = 3.047$, $P \leq 0.005$) (Figure 21).

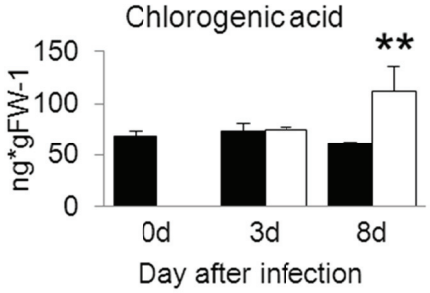


Figure 21. Induction of chlorogenic acid in *Nicotiana attenuata* plant upon *F. solani* Utah 4 infection. Infection setup and growth condition for both infections are described in detail in the caption of Figure 15. Plants were harvested at three time points, the first time point was at the day of the infection (0d), the second time point was 3 dai (3d) and the third time point was 8 dai (8d). Five biological replicates (one seedling per replicate) were used. Secondary metabolite concentration was calculated nanogram per gram of fresh weight (ng*gFW^{-}1). Black and white bars present chlorogenic acid levels of non-infected (black) and infected (white) plants. Asterisks indicate significant differences between infected and non-infected plants at a given time point (one-way ANOVA with LSD posthoc test, $P \leq 0.05$).

5.10 Influence of JA and SA signaling and secondary metabolite production on the disease progression during *Fusarium brachygibbosum* Utah 4 and *Alternaria alternata* Utah 10 infection

To further explore the role of the induced phytohormones (SA and JA) and secondary metabolites (nicotine, CA, CCA, DCS and rutin) in *N. attenuata´*s defense against the Utah fungal pathogens, *N. attenuata* plants which are compromised in JA, SA and secondary metabolite biosynthesis and JA-Ile perception were used for an infection assay with *F. brachygibbosum* Utah 4 and *A. alternata* Utah 10. The infection method used in this assay was the one described above for the second infection assay. *F. solani* Utah 4 was not used at all for this infection assay because of above mentioned safety issues and risk assessment reasons. Chlorosis, necrosis, wilting, growth performance (rosette diameter and stalk length), flower morphology as well as bolting and flowering time served again as criteria to assess disease progression. In addition, the ability of plants to recover was observed. All stably transformed *N. attenuata* lines used in this experiment are listed in Supplemental Table 1 and their characteristic properties providing the reason why they had been chosen for this

experiment are described in the material and methods part. For better understanding, all products from genes involved in JA biosynthesis and signaling that are stably silenced in the plant lines used for this experiment are illustrated in red in Figure 22 below

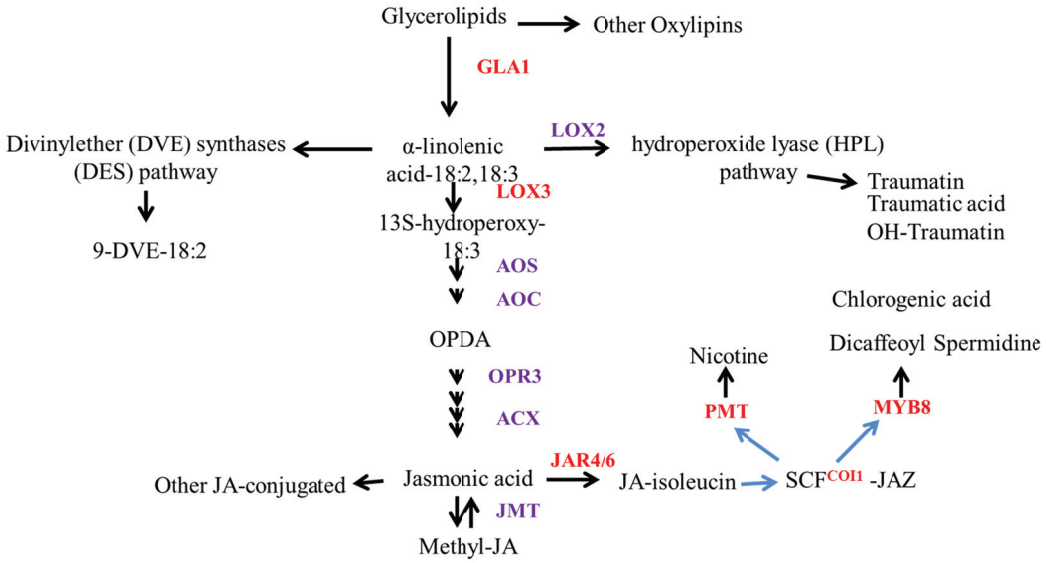


Figure 22. Proteins related to jasmonate biosynthesis and signaling pathway in *N. attenuata*. All proteins in red font were silenced by small RNA interference (RNAi) technique in *N. attenuata* genotypes used for an infection assay. Details for those lines including the vector constructs used to generate them are listed in Supplemental Table 1. In *N. attenuata*, α-linolenic acid (18:3) is released by glycerolipase 1 (GLA1) from chloroplast membrane glycerolipids (Bonaventure *et al.*, 2011), oxidized by lipoxygenase 3 (LOX3) at C13 to form 13S- hydroperoxy-18:3 (13S-OOH-18:3) (Halitschke and Baldwin, 2003). 13S-OOH-18:3 is the substrate for allene oxide synthase (AOS) that forms 12,13-epoxy-18:3 which is subsequently cyclized to 12-oxo-phytodienoic acid (OPDA) by allene oxide cyclase (AOC) (Schaller, 2001). OPDA is then reduced by OPDA reductase 3 (OPR3) (Breithaupt *et al.*, 2006). The reduced OPDA (OPC-8:0) undergoes three cycles of ß-oxidation, involving acetyl-CoA transferase 1 (ACX1) (Pedersen and Henriksen, 2005) and finally forming (+)-7-iso-jasmonic acid (JA). JA can be further modified, for example, by jasmonyl O-methyl transferase (JMT) to form methyl-jasmonic acid (MeJA) or conjugated to isoleucine (Ile) by JASMONATE RESISTANT 4 and 6 (JAR4 and JAR6) to form JA-Ile (Wang *et al.*, 2008). JA-Ile activates the SCF^{COI1}-JAZ complex which transcriptionally activates genes involved in the biosynthesis of defense molecules, such as the gene encoding putrescine N-methyltransferase (PMT) in nicotine biosynthesis (Zavala *et al.*, 2004) , the gene encoding MYB8 transcription factor involved in the biosynthesis of phenyl-propanoid compounds such as caffeoylputrescine (CP), decaffeoylspermidine (DCS) and some other phenolic compounds such as chlorogenic acid (Kaur *et al.*, 2010)

In order to explore the role of SA in plant's defense against Utah fungi, ovNahG line which overexpresses a salicylate hydroxylase from *Pseudomonas putida* (NahG), an enzyme which inactivates SA signaling in *N. attenuata* (Gilardoni *et al.*, 2011) was used. In addition,

N. attenuata Arizona wild type (AzWT) plants which exhibits large differences in JA signaling induced by the specialist herbivore *Manduca sexta* compared to Utah WT (UtWT), including herbivore-induced JA, JA-Ile and basal levels of secondary metabolites nicotine, chlorogenic acid and rutin (Wu *et al*., 2008) was also included in the infection assay.

As a result from this infection assay, irgla1 and aslox3 lines suffered most from *F. brachygibbosum* infection compared to UtWT and the other transgenic plants (Figure 26 A). Infected irgla1 and aslox3 plants were the first ones showing disease symptoms like wilting, chlorosis and necrosis. Especially wilting appeared very early at 8 dai in infected aslox3 plants (Figure 23 D) and at 12 dai in infected irgla1 plants (Figure 23 C). The percentage of wilted plants increased dramatically (exponentially) over time for the infected irgla1 plants, but increased quite linear for aslox3 plants. After the appearance of wilting, infected irgla1 plants turned chlorotic and dried out very fast before necrosis could be observed. After wilting started, aslox3 plants developed not only chlorosis but also necrosis, which occurred at the late stage of disease symptom development. As a consequence of this severe disease progression, at 44 dai, irgla1 had the highest percentage of dead plants compared to all other genotypes (56%) (Figure 26 B). At that time aslox3 had the highest percentage of diseased and dead plants compared to the other genotypes (summed up to 72%). Some other infected plants continued growing, however irgla1 and aslox3 plants were in average significantly smaller than WT (*t* test, $P < 0.02$) (Figure 24 C and D). The percentage of infected plants with a rosette diameter of less than 10 cm at 29 dai was 56% for aslox3 and 20% for irgla1. Percentage of infected plants that had stalk heights shorter than 40 cm at the same time was 36% for aslox3 and 16% for irgla1 (Figure 26 B). All surviving plants showed typical crown rot of *Fusarium* infection even though they appeared more or less healthy at the first glance by looking only at their above-ground plant parts. Flowering time and bolting time of those two transgenic lines was also delayed much more than in other transgenic lines, especially for irgla1 which was delayed in bolting for 2 days and in flowering for even almost 5 days. In a nutshell, irgla1 and aslox3 were the two most susceptible transgenic lines to *F. brachygibbosum* Utah 4 infection in comparison to other transgenic lines such as ircoi1, irjar4/6, irpmt, irmyb8, ovNahG and both non-transgenic *N. attenuata* accessions (Utah and Arizona wildtypes).

Other transgenic lines (irjar4/6, irpmt and irmyb8) were also more susceptible to *F. brachygibbosu*m Utah 4 infection than UtWT, but less than irgla1 and aslox3. Those lines started to show wilting after 16 to 18 days infection (Figure 23 F, G and H), followed by the development of chlorosis and necrosis. Till 44 dai, 8% of infected plants of irjar4/6 and irpmt were dead and 24 % of all infected plants showed disease symptoms (Figure 26 B). None of

the infected irmyb8 plants died until 44 dai, but 48% of them showed disease symptoms. Surviving infected plants developed slower than non-infected plants in all three lines; infected plants remained significant smaller in rosette diameter as well as stalk height (t test, $P < 0.02$) (Figure 24, 25, F, G, H). Bolting time and flowering time therefore was delayed for about 1 day in irjar4/6, irpmt, infected irmyb8 plants, except for flowering time of irpmt which was almost not different to non-infected plants.

Interestingly, infected ircoi1and ovNahG plants showed no significant difference in disease symptom development compared to UtWT (Figure 26 A). At 44 dai, 84% of all infected ovNahG and UtWT plants were asymptomatic, meaning none of them showed any observable disease symptoms, especially true for the infected ircoi1 plants (88% asymptomatic) (Figure 26 B). At 44 dai, total percentage of dead and diseased plants was 16% for infected UtWT and ovNahG plants, whereas it was only 12% for ircoi1. Moreover, growth parameters of infected ircoi1 plants were almost equal to those from non-infected plants (Figure 24, 25 E). In contrast, infected ovNahG plants were remaining significantly smaller in rosette diameter and shorter in stalk height compared to non-infected plants (Figure 24, 25 I). And infected UtWT plants were significantly smaller compared to uninfected ones in rosette diameter at late stage of plant development (Figure 24 A). This result indicated that, although there was not a large difference between *F. brachygibbosum* Utah 4 infected ovNahG and ircoi1 plants compared to UtWT, a variation in certain aspects of their response to the pathogen was still observable: ircoi1 genotype seemed to be slightly more resistant to *F. brachygibbosum* Utah 4 than UtWT, while ovNahG seemed to be slightly more susceptible to *F. brachygibbosum* 4 than UtWT.

There was no significant difference in disease symptoms like chlorosis, necrosis and wilting at 34 dai between infected Utah and Arizona WT (Figure 26 A). There was only a small difference between Utah and Arizona WT in rosette diameter. Infected AzWT showed significantly smaller rosette diameter than non-infected plants from 17 dai on (Figure 24 B), but UtWT only started to show this reduction in rosette diameter at 35 dai (Figure 24 A). This indicates that Arizona and UtWT do not differ a lot in their response to *F. brachygibbosum* Utah 4, however these little differences still lead to changes in plant growth.

For *F. brachygibbosum* Utah 4 no changes in flower morphology due to the infection could be observed for WT as well for the transgenic lines (Figure 26 C).

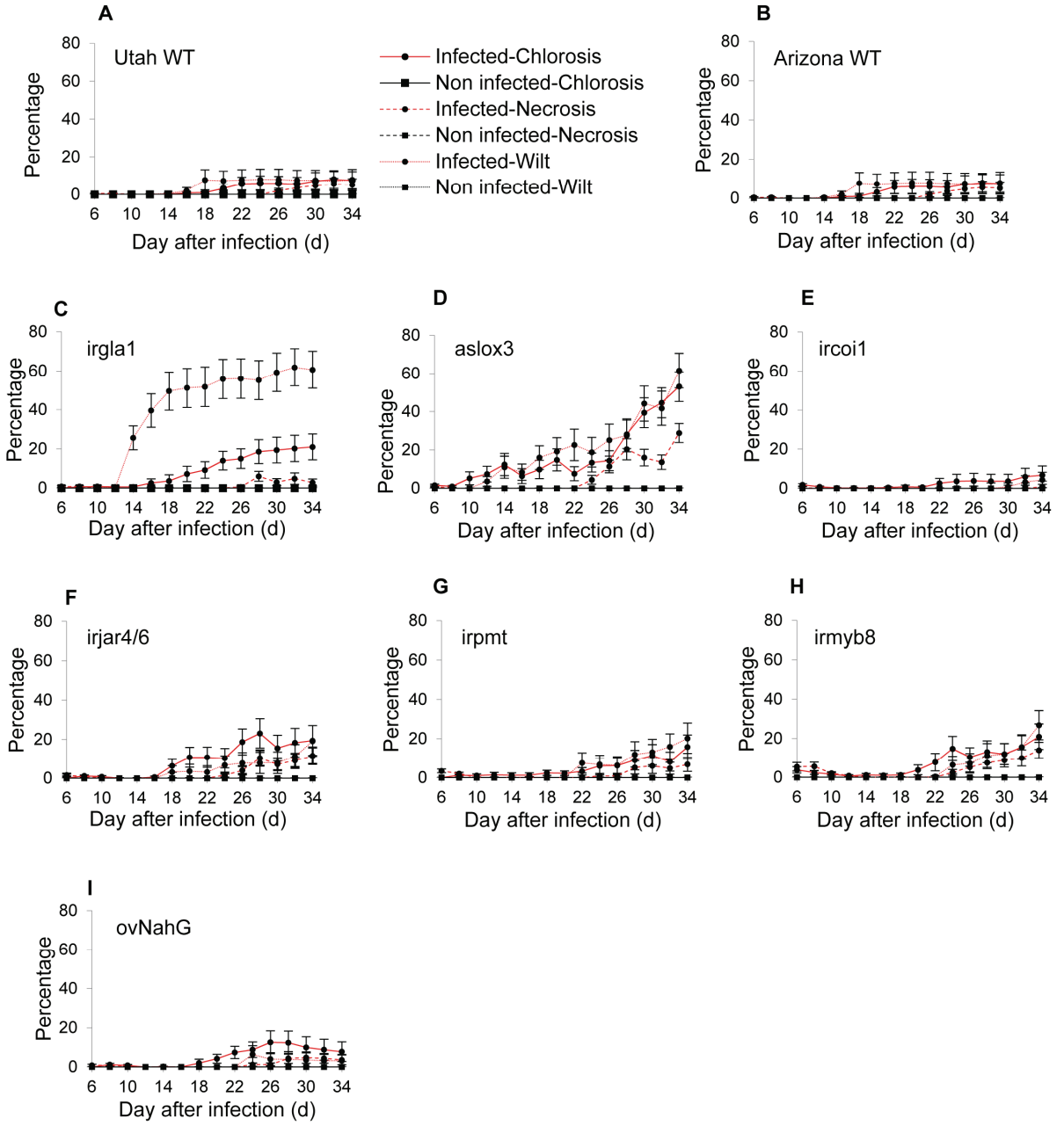


Figure 23. Development of chlorosis, necrosis and wilting of *N. attenuata* UtWT (A), AzWT (B), irgla1 (C), aslox3 (D), ircoi1 (E), irjar4/6 (F), irpmt (G), imyb8 (H), ovNahG (I) plants in response to *F. brachygibbosum* Utah 4 infection over time. *F. brachygibbosum* Utah 4 infected plants (red lines) were compared to non-infected plants (black lines), presenting the average percentage of chlorosis (solid lines), necrosis (dotted lines) and wilting (dashed lines)

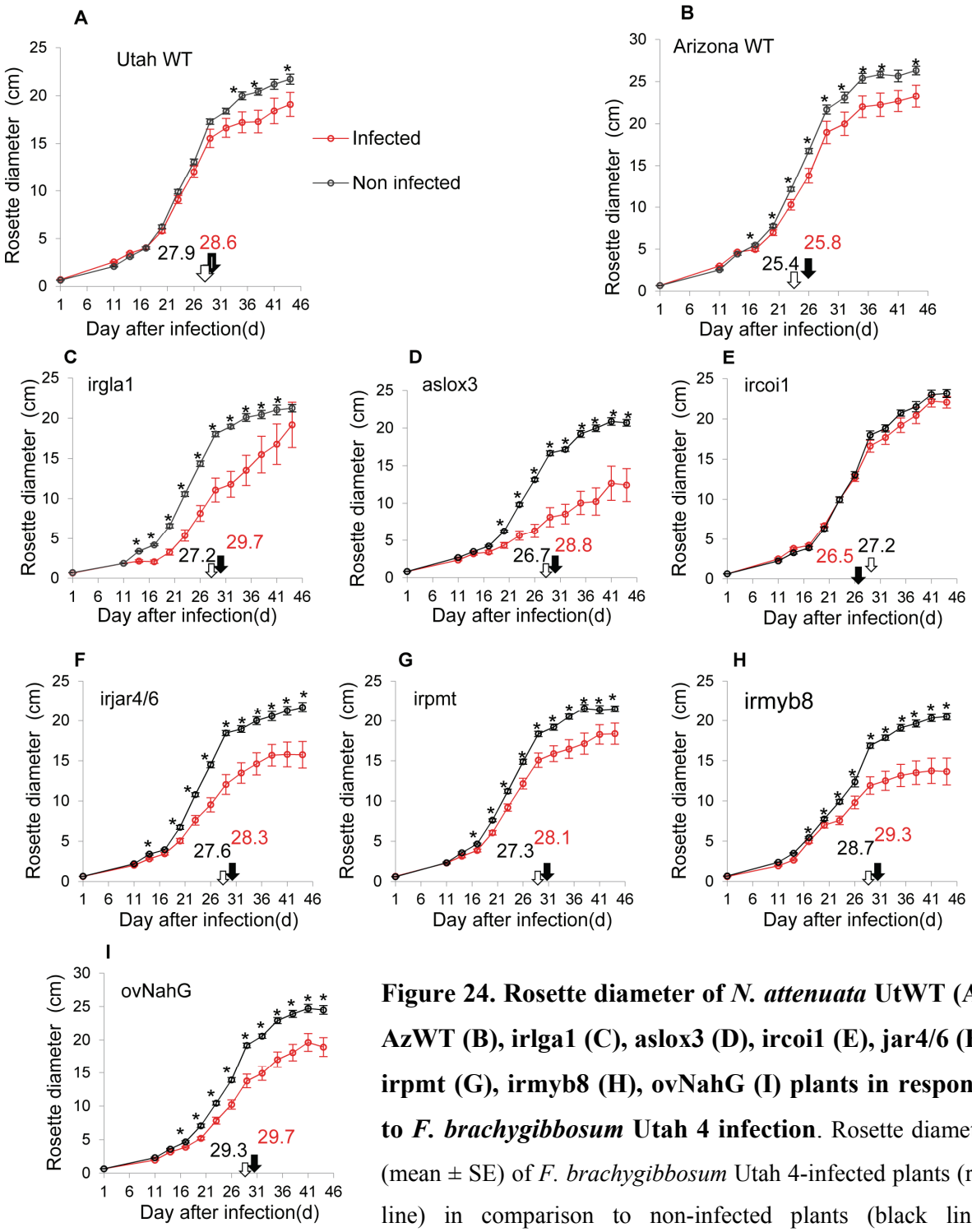


Figure 24. Rosette diameter of *N. attenuata* UtWT (A), AzWT (B), irlga1 (C), aslox3 (D), ircoi1 (E), jar4/6 (F), irpmt (G), irmyb8 (H), ovNahG (I) plants in response to *F. brachygibbosum* Utah 4 infection. Rosette diameter (mean ± SE) of *F. brachygibbosum* Utah 4-infected plants (red line) in comparison to non-infected plants (black line). Numbers on top of the downwards pointing arrows show the average bolting time in infected plants (red) and non-infected plants (black). Asterisks indicate significant differences between infected and non-infected plants (*t* test, $P \leq 0.05$).

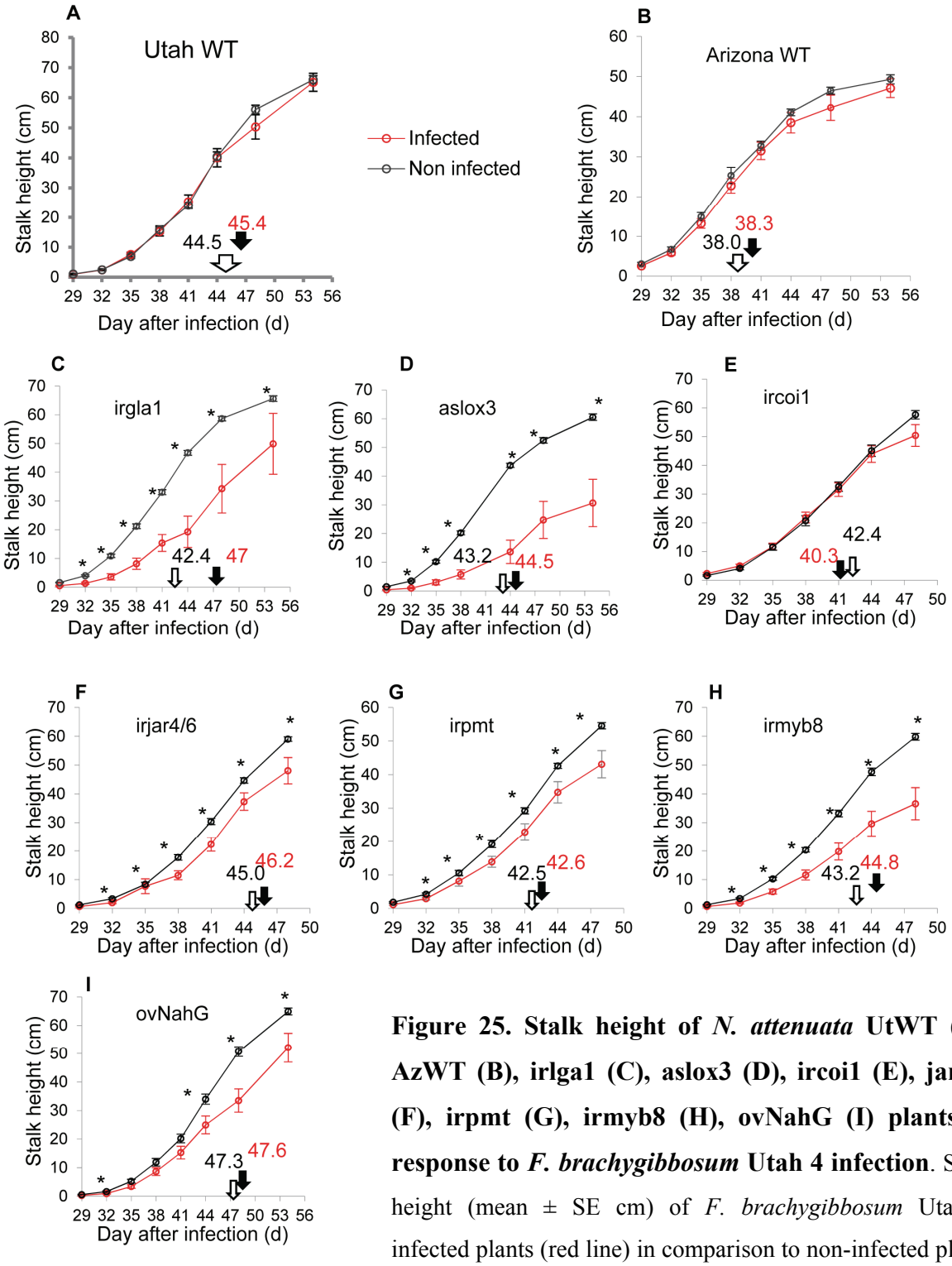
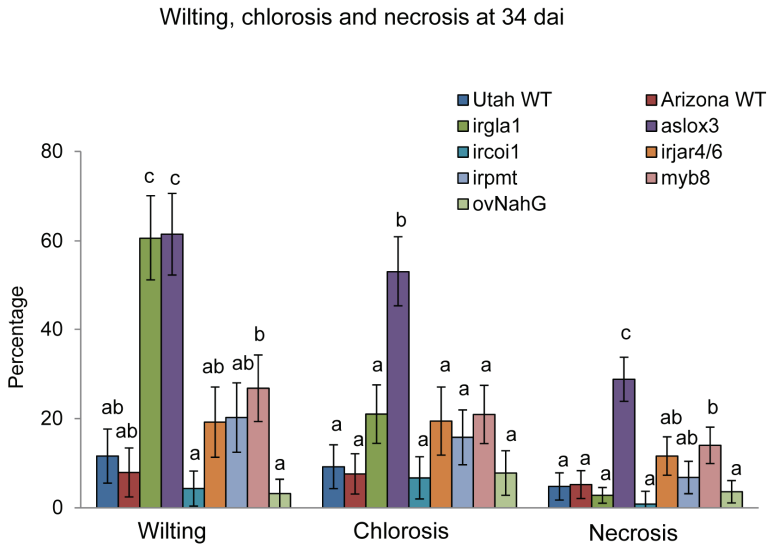


Figure 25. Stalk height of *N. attenuata* UtWT (A), AzWT (B), irlga1 (C), aslox3 (D), ircoi1 (E), jar4/6 (F), irpmt (G), irmyb8 (H), ovNahG (I) plants in response to *F. brachygibbosum* Utah 4 infection. Stalk height (mean ± SE cm) of *F. brachygibbosum* Utah 4 infected plants (red line) in comparison to non-infected plants (black line). Numbers on top of the downwards pointing arrows show the average flowering time in infected plants (red) and non-infected plants (black). Asterisks indicate significant differences between infected and non-infected plants (*t* test, $P \leq 0.05$).

A



B

Line	dead	diseased	asymptomatic	RD ≤ 10cm	RD >10cm	SH ≤ 40cm	SH >40cm
	44 dai			29 dai		54 dai	
UtWT	4	12	84	20	80	4	96
AzWT	4	8	88	12	88	8	92
irgla1	56	12	32	20	80	16	84
aslox3	36	36	28	56	44	36	64
ircoi1	4	8	88	4	96	4	96
irjar4/6	8	24	68	32	68	16	84
irmyb8	0	48	52	28	72	48	52
irpmt	8	24	68	12	88	28	72
ovNahG	0	16	84	16	84	28	72

C

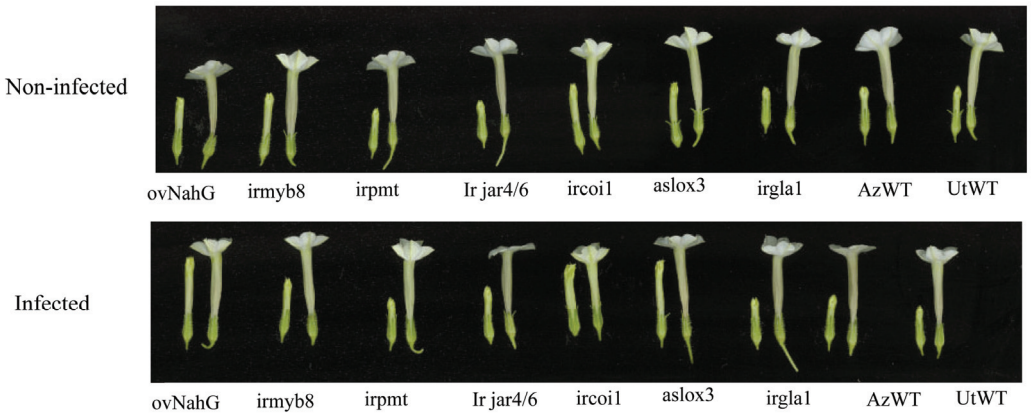


Figure 26. Disease symptom quantification of *N. attenuata* WT and transgenic plants in response to *F. brachygibbosum* Utah 4 infection. A. Percentage of the disease symptoms wilting, chlorosis and necrosis of *F. brachygibbosum* Utah 4-infected UtWT (dark blue) , AzWT (red), irgla1 (green), aslox3 (purple), ircoi1(sky-blue), irjar4/6 (orange), irpmt (light blue), irmyb8 (pink) and ovNahG (light green) plants 34 days after infection. Error bars represent standard deviation from

25 infected replicates. Different letters (a-c) indicate significantly different data groups determined by ANOVA ($P \leq 0.05$). **B**. The table shows percentages of dead, diseased and asymptomatic plants at 44 dai, as well as the percentage of infected plants with a rosette diameter smaller than 10 cm (RD ≤ 10cm) or bigger than 10 cm (RD > 10cm) at 29 dai and the percentage of infected plants which have stalk height shorter than 40 cm (SH ≤ 40cm) or higher than 40 cm (SH >40cm) at 54 dai. **C**. Flower morphology of infected and non-infected plants of all wild type and transgenic lines used in the infection with *F. brachygibbosum* Utah 4.

Unlike *F. brachygibbosum* Utah 4 infection, *A. alternata* Utah 10 infection did not result in major differences in observable disease symptoms such as chlorosis, necrosis and growth parameters between the wild type accessions and the tested transgenic lines. Infected plants started to show weak chlorosis at 4 dai which increased quickly between 5 to 10 days after infection. Necrotic lesions hardly occurred at any infected plant. Again, no wilting appeared at *A. alternata* Utah 10-infected plants. At 10 to 15 days after infection, all infected plants recovered and disease symptoms like chlorosis and necrosis were almost disappeared (Figure 28). Since maximum chlorosis was recorded at 8 dai for most of the genotypes, a comparison between infected plants of UtWT, AzWT and transgenic lines was made at that time point as shown in Figure 27. Among all tested transgenic lines, only infected ovNahG plants had a similar chlorosis level to infected UtWT. Infected plants of all other transgenic lines showed less chlorosis than UtWT (Figure 28). In addition, the infection with *A. alternata* Utah 10 had little impact on stalk height of UtWT and ovNahG at early stage of elongation (Figure 30 A, I) whereas it caused a greater reduction in rosette diameter on infected ovNahG plants (Figure 29 I) comparing to it in infected UtWT plants (Figure 29 A). Moreover, *Alternaria* infection caused delay in bolting and flowering time of ovNahG plants but not in UtWT. This result indicates that ovNahG plants are more affected by *A. alternata* Utah 10 at rosette stage.

All infected aslox3, ircoi1, irjar4/6, irmyb8 and irpmt plants showed less chlorosis and necrosis than infected UtWT plants at 8 dai (Figure 28). Consequently, less fungus-mediated effects in growth of those infected lines were expected. Surprisingly, the infection with *A. alternata* Utah 10 did have different effects on plant growth for those lines either at rosette or stalk elongation stage as detailed in Figure 29 and 30 for each line. For instance, infected irlox3, ircoi1, irjar4/6 and irpmt plants had smaller rosette diameter in comparison to non-infected plants at rosette stage (Figure 29). In addition, infected irjar4/6 and irpmt plants showed also a strong reduction in stalk height compared to non-infected plants (Figure 30). However, stalk length in irmyb8 plants was reduced only for limited time (39 to 44 dai)

during the infection. This variety of changes suggested that: in spite of a weak disease manifestation, there were still sufficient characters to distinguish between the different transgenic lines.

Interestingly, infected irgla1 plants showed slightly stronger chlorosis than the other transgenic lines, but no changes in growth. Infected AzWT plants showed almost no disease symptoms at all. Therefore, it is feasible to conclude that irgla1 and AzWT plants are more resistant to *A. alternata* Utah 10 infection than UtWT plants.

In summary, by using different transgenic lines compromised in JA-Ile biosynthesis (irgla1, aslox3 and irjar4/6), perception (ircoi1), secondary mmetabolite production (irpmt and irmyb8) and SA biosynthesis (ov-nahG), it could be demonstrated that both Utah fungal pathogens (*F. brachygibbosum* Utah 4 and *A. alternata* Utah 10) can be used for pathogen infection assays to discrimate between different *N. attenuata* genotypes compromised in certain disease-relevant traits. The results from these infection assays further demonstrated that the biosynthesis of oxylipins derived from the JA-biosynthesis pathway and the production of certain secondary metabolites (nicotine, rutin, CA, CCA, DCS) but not JA-Ile perception, play a crucial role in plant defense against *F. brachygibbosum* Utah 4. Moreover, SA plays an important role in plant defense against *A. alternata* Utah 10.

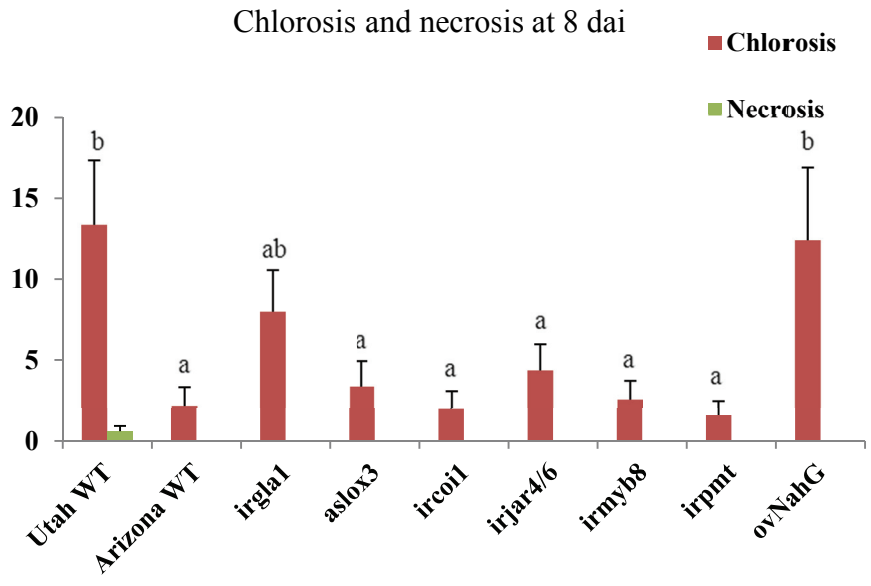


Figure 27 . Percentage of chlorosis (red bar) and necrosis (green bar) from *A. alternata* Utah 10-infected UtWT, AzWT, irgla1, aslox3, ircoi1, irjar4/6, irpmt, irmyb8 and ovNahG plants at 8 days after infection. Error bar represent standard deviation from 25 replicates.

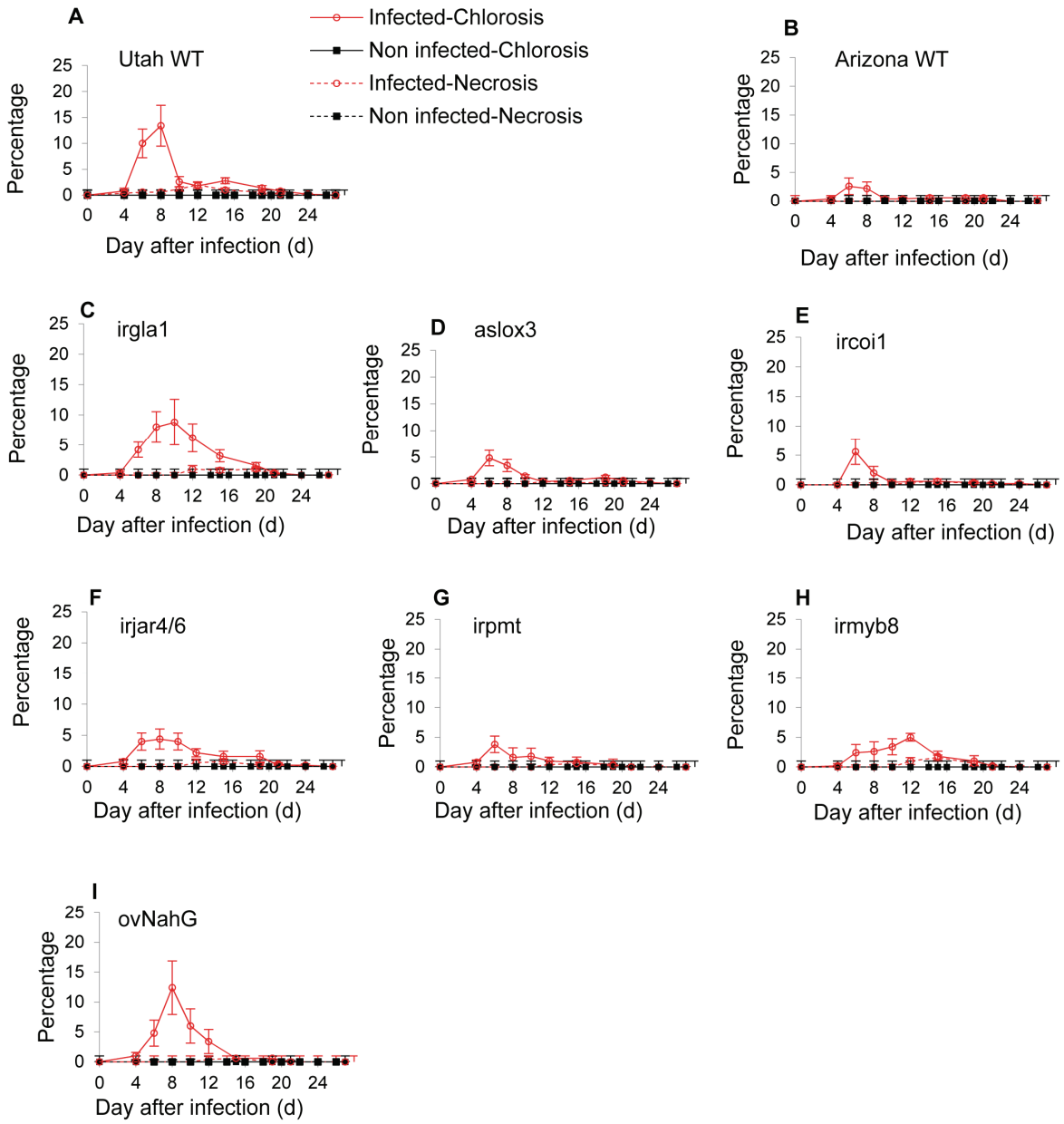


Figure 28. Development of chlorosis, necrosis and wilting of *N. attenuata* UtWT (A), AzWT (B), irgla1 (C), aslox3 (D), ircoi1 (E), irjar4/6 (F), irpmt (G), imyb8 (H), ovNahG (I) plants in response to *A. alternata* Utah 10 infection over time. *A. alternata* Utah 10 infected plants (red lines) were compared to non-infected plants (black lines), presenting the average percentage of chlorosis (solid lines), necrosis (dotted lines) and wilting (dashed lines)

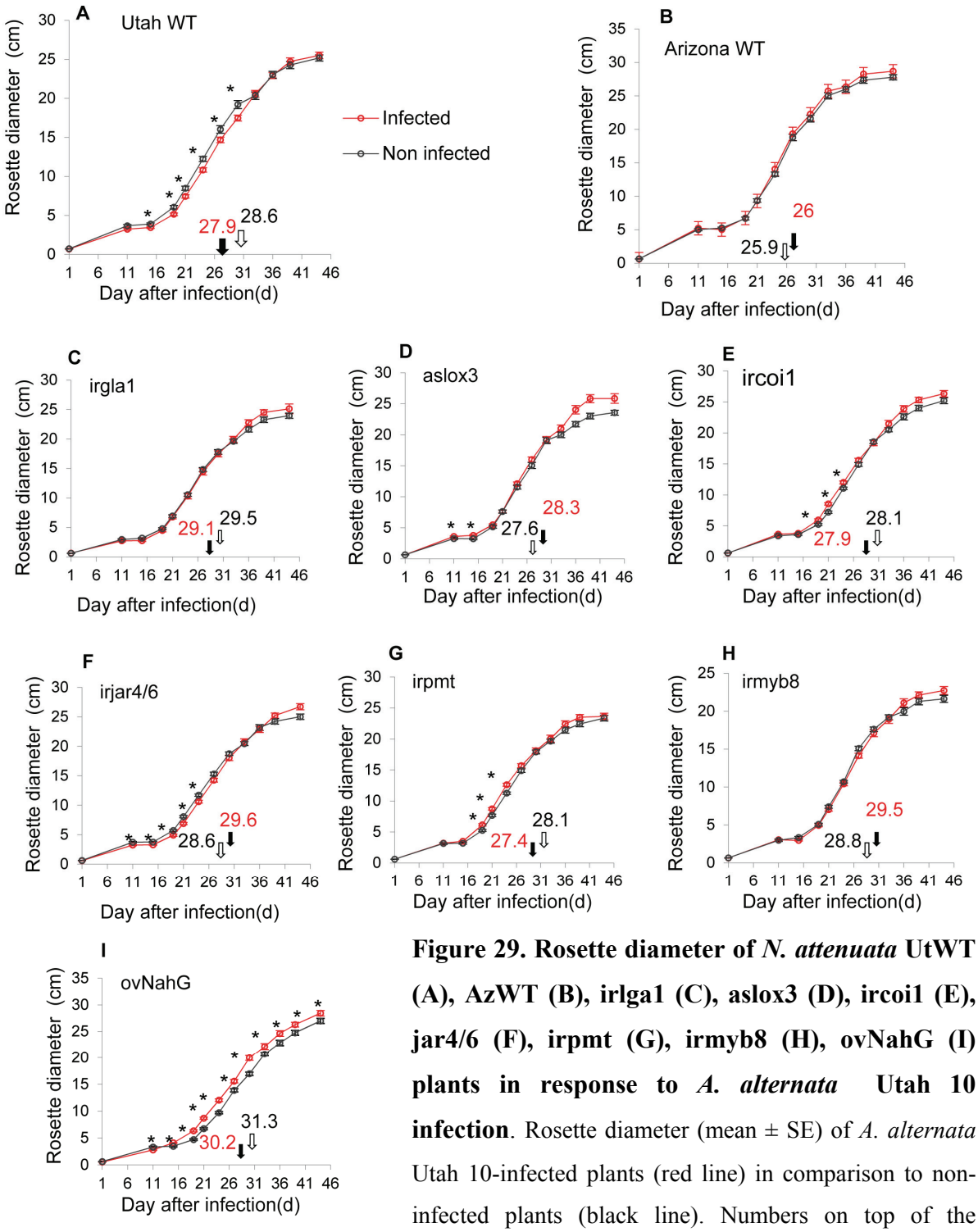


Figure 29. Rosette diameter of *N. attenuata* UtWT (A), AzWT (B), irlga1 (C), aslox3 (D), ircoi1 (E), jar4/6 (F), irpmt (G), irmyb8 (H), ovNahG (I) plants in response to *A. alternata* Utah 10 infection. Rosette diameter (mean ± SE) of *A. alternata* Utah 10-infected plants (red line) in comparison to non-infected plants (black line). Numbers on top of the downwards pointing arrows show the average bolting time in infected plants (red) and non-infected plants (black). Asterisks indicate significant differences between infected and non-infected plants (*t* test, $P \leq 0.05$).

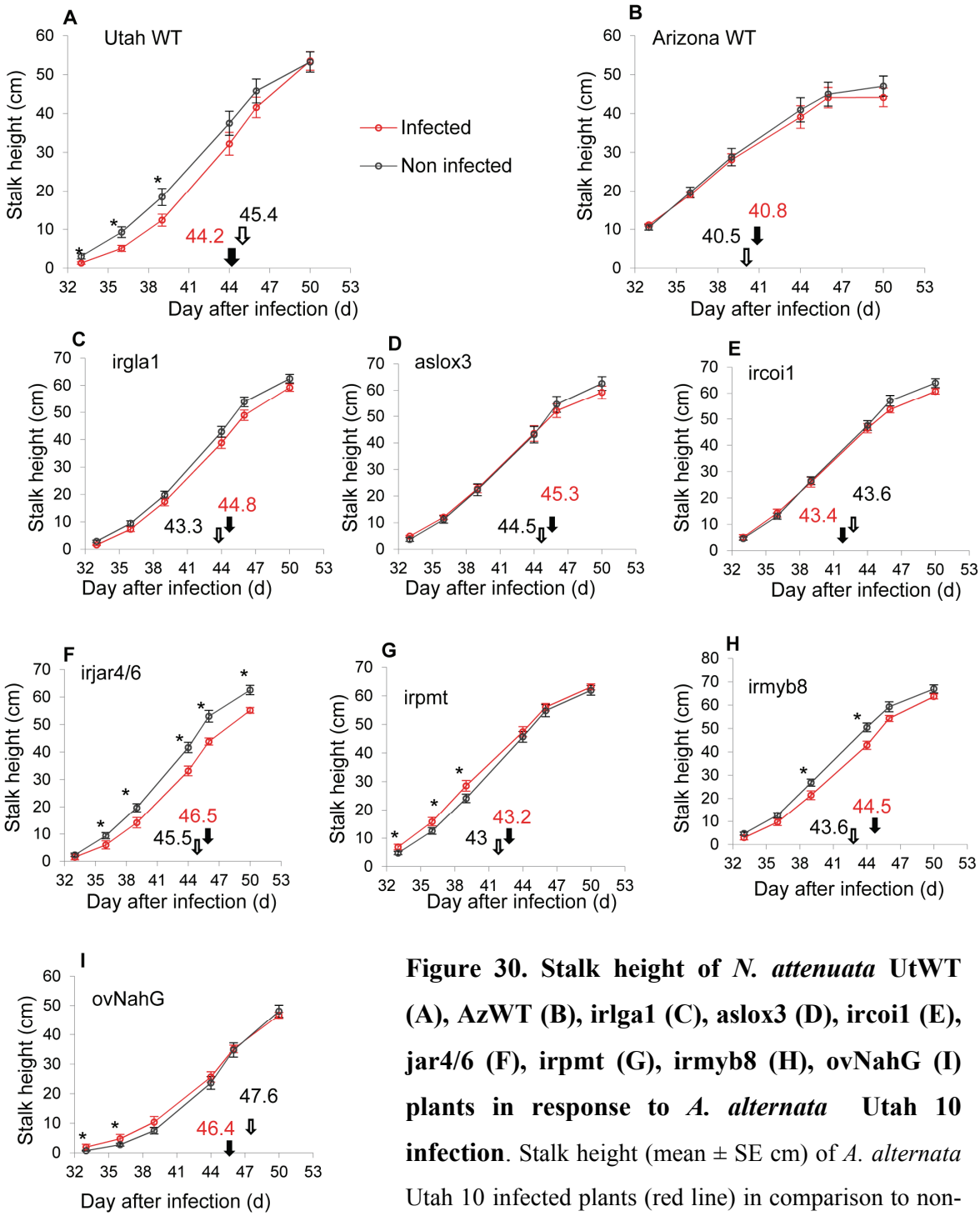


Figure 30. Stalk height of *N. attenuata* UtWT (A), AzWT (B), irlga1 (C), aslox3 (D), ircoi1 (E), jar4/6 (F), irpmt (G), irmyb8 (H), ovNahG (I) plants in response to *A. alternata* Utah 10 infection. Stalk height (mean ± SE cm) of *A. alternata* Utah 10 infected plants (red line) in comparison to non-infected plants (black line). Numbers on top of the downwards pointing arrows show the average flowering time in infected plants (red) and non-infected plants (black). Asterisks indicate significant differences between infected and non-infected plants (*t* test, $P \leq 0.05$).

5.11 Large variability in the defense response of different *Nicotiana attenuata* accessions using a detached leaf assay with *F. solani* Utah 4

In a previous study, the comparison between two *N. attenuata* accessions revealed large differences in herbivore-induced responses including phytohormone and secondary metabolite levels (Wu *et al*., 2008). Taken together with the random distribution of diseased plants within the native population in Utah 2011, it was hypothesized that plants within and between different native populations of this species may differ a lot in their response to the native pathogens. To falsify this hypothesis, detached leaves from 32 different accessions were used for inoculation with *F. solani* Utah 4. Three fungal agar plugs of *F. solani* Utah 4 were placed on one leaf and kept in moist condition as described in material and method. Responses were recorded at 3, 4 and 5 days after inoculation. The "product" value representing the level of response of each accession leaf is shown in Figure 31 C and detailed data is shown in Supplemental Table 2. Fifteen accessions showed hyper-resistance, 11 accessions showed resistance to the pathogen whereas 16 and 23 accessions were susceptible and hyper-susceptible to the pathogen, respectively. This strong separation between *N. attenuata* accessions in their response to the pathogen indicated the diversity of natural populations in their defense against native pathogens.

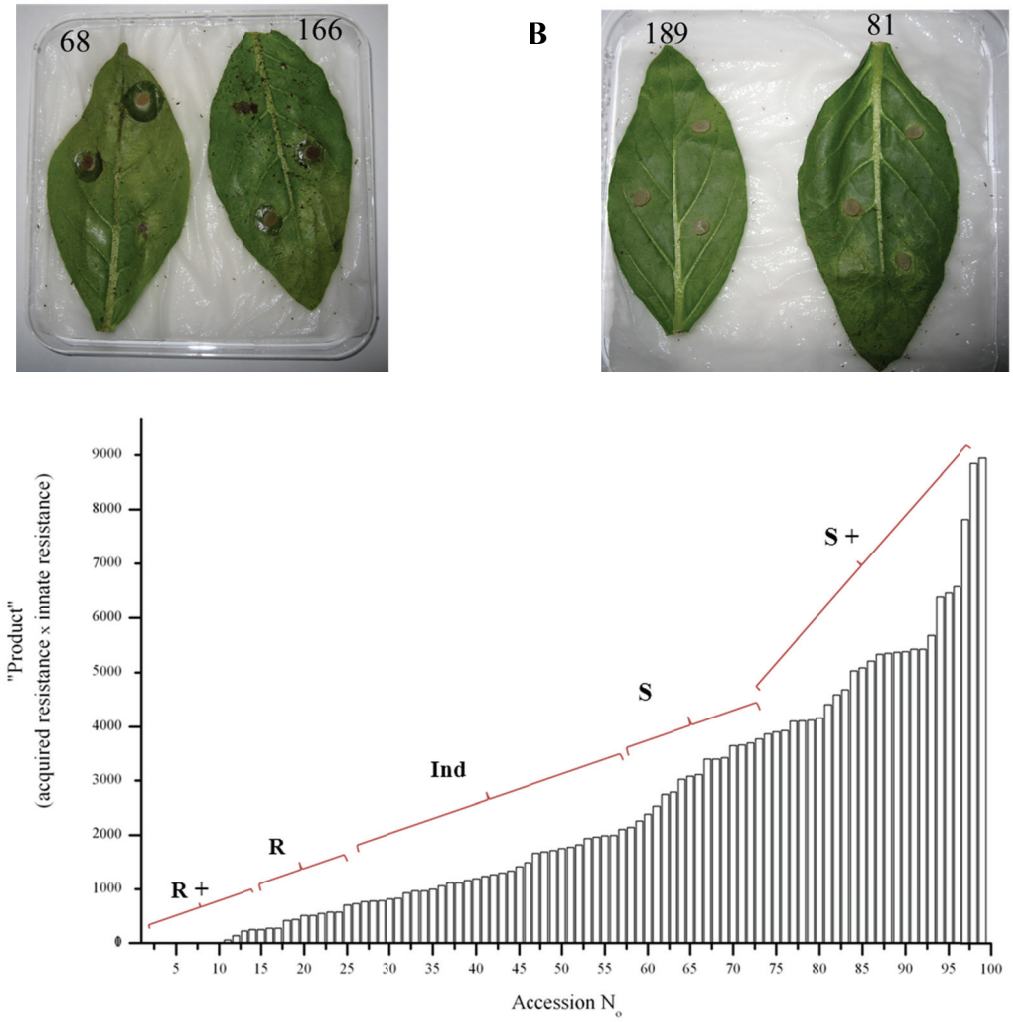


Figure 31. Response strength of different *N. attenuata* accessions towards *F. solani* Utah 4. **A**. Hypersensitive response and necrotic lesions on inoculated detached leaves at 3 days after inoculation. **B**. Chlorosis on inoculated detached leaves at 5 days after inoculation. **C**. Products (response values) of each *N. attenuata* accession to *F. solani* Utah 4-infection at 5 days after inoculation. 99 plants were randomly picked from 31 different accessions. One fully expanded leaf per plant was used with three replicates per leaf. Three agar plugs containing actively growing fungal mycelium were placed on three different sites of a detached leaf. Plates were maintained in moist condition (70% relative humidity) and kept at 25°C with a 14 h photoperiod. Diameter of hypersensitive response lesions (as indicator for induced resistance) and chlorotic lesions (resembling rather basal resistance) were measured at 3, 4 and 5 days after inoculation. Percentage of hypersensitive response and chlorosis was then multiplied to obtain a "product" value. This product value was used to evaluate the response of each accession using the following categories: "Product" ≤ 247: Hyper-resistance (R^+); 247 < "Product" ≤ 728: Resistance (R); 728 < "Product" ≤ 2384: Indifferent (Ind); 2384 < "Product" ≤ 3930: Susceptible (S); "Product" > 3930: Hyper- susceptible (S^+).

5.12 Influence of *F. brachygibbosum* Utah 4 and *Alternaria alternata* Utah 10 infection on the colonization by the mutualistic fungus *Piriformospora indica*.

Another infection assay was performed with *N. attenuata* seedlings that had been preinoculated with the mutualistic fungus *Piriformosspora indica* on plate. After 12 days, the seedlings were either inoculated with *A. alternata* Utah 10 or *F. brachygibbosum* Utah 4. Seedlings either uninoculated or inoculated with *P. indica* that were treated only with $MgSO_4$ solution served as control. At 8 dai, seedlings were harvested and used for genomic DNA extraction. 15 ng total genomic DNA was then used to measure colonization by all three fungi species by quantitative RT-PCR (qPCR) using species-specific primers. These primers either had been already published to be appropriate for the quantification of fungal colonization (*A. alternata* and *P. indica*) (Schuhegger *et al*., 2006; Deshmukh *et al*., 2006) or designed based on species-specific DNA sequences (*F. brachygibbosum* Utah 4). Specificity of the primers used for *A. alternata* Utah 10 and *F. brachygibbosum* Utah 4 quantification was confirmed before by PCR (Supplemental Figure 3), however the *A. alternata* Utah 10 primers turned out to be unspecific when using for qPCR. qPCR quantification of *F. brachygibbosum* Utah 4 colonization failed and no amplification could be observed. However, quantification of *P. indica* by qPCR worked well and gave an interesting result: After infection with the two Utah fungal pathogens, colonization of *N. attenuata* plants by *P. indica* increased approximately 3-fold compared to the medium control (see Figure 32).

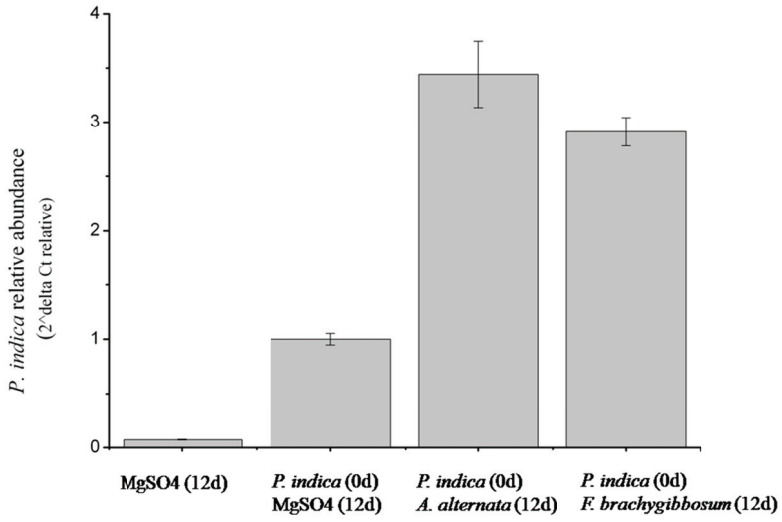


Figure 32. Effects of Utah pathogenic fungi on the colonization by the mutualistic fungus *P. indica*. DNA was extracted from entire *N. attenuata* WT plants which were infected for 8 days with either *A. alternata* Utah 10 or *F. brachygibbosum* Utah 4. qPCR based on the relative abundance of the Pi-EF1a compared to the Na-EF1a gene (n=18 to 20; bars = relative abundance ± S.E.)

6 Discussion

Up to now this is the first study on *N. attenuata* dealing with a fungal disease outbreak in its natural habitat, the Great Basin desert, a part from southwestern Utah, USA. While trying to identify the causal agents of this disease outbreak, three fungal species (*F. solani*, *F. brachygibbosum* and *A. alternata* which are able to infect *N. attenuata* under laboratory conditions were found. Setting up an experimental infection to perform proper infection assays using these pathogens on the wild tobacco plants will be most likely of immense value for future research. The three fungi species were then further characterized regarding the response that they induce in the plant not only morphologically (chlorosis, necrosis, wilting, root crown rot and various other growth parameters), but also on phytohormone and secondary metabolite level. Furthermore, this study demonstrated that two of those fungal pathogens (*F. brachygibbosum* Utah 4 and *A.alternata* Utah 10) can be used as potential screening tools to compare different *N. attenuata* genotypes regarding their response to those pathogens. A first attempt to study the effect of prior inoculation of *N. attenuata* plants with the mutualistic fungus *Piriformospora indica* on the performance of the two fungal pathogens failed, however, this experiment will be repeated in near future using new knowledge gained.

6.1 Identification of the pathogenic fungi isolated from *N. attenuata*

The fungi isolated from infected *N. attenuata* leaves belong to the gernera *Fusarium* and *Alternaria* which are two phytopathogenic fungi groups known to typically occur also in desert environment (Rotem, 1998; Hine, 1999). Regarding the genus of *Fusarium* , six isolates of *F. solani* and four isolates of *F. brachygibbosum* were classified by ITS sequencing and confirmed by microscopy. Members of the genus of *Fusarium* and in particular species of *F. solani* are well-known for their ability to infect plant roots, entering the plants vascular system and to cause wilt and crown/root rot disease in a wide range of hosts distributed among 66 plant families comprising also crop plants like soybean (Hartman *et al*., 1995), tomato (Vawdrey and Peterson, 1988) and papaya (Quimio, 1976). However, these symptoms were not found in the diseased native *N. attenuata* plants in Utah 2011. Very little is known about *F. brachygibbosum*. It can be isolated from undisturbed rather than from cultivated soil and predominantly distributed in soil of the warm temperate zone with low rainfall (Adler and Lew, 1995). This conditions are similar to the climatic conditions in the desert of Utah which is a land of extreme temperatures and rainfall mainly occurring in spring

and winter with only around 305 mm (Pellant *et al*., 2004). Only few reports of this fungus species related to plants were published so far. *F. brachygibbosum* was originally described by Padwick in 1995 from a specimen of *Sorghum vulgare* in India. In 1993, *F. brachygibbosum* was reported in common bean (*Phaseolus vulgaris*) in Sudan where it was not a plant pathogen (EL Tayeb Mohamed EL Tayeb, 1993). In 2012 it was reported for the first time as a new root pathogen of date palm (Al-Sadi *et al*., 2012). However, the interaction of this fungus with plant remains completely unknown.

12 *Alternaria* isolates were also found in infected *N. attenuata* leaf samples. *Alternaria*. is known as a genus comprising saprophytic and parasitic species commonly found in decaying plant material or in soil. Species from this genus can infect over 380 host plants as recorded in the USDA Systematic Botany and Mycology Fungus-Host Distribution Database (http://nt.arsgrin.gov/fungaldatabases/index.cfm). Solanaceous crops are also known to be infected by *Alternaria sp.* including eggplant, pepper, potato and tomato (Andersen and Frisvad, 2004). *Alternaria alternata* (Fries) Keissler is a parasitic fungus which causes brown spot disease of senescing tobacco (*Nicotiana tabacum* L.) leaves (Duan *et al*., 2010). The typical disease symptoms caused by these fungi are necrotic lesions surrounded by chlorotic halos and sometimes the lesion is covered with a fine, black and fuzzy growing mycelium of *Alternaria* sporulating on the dying host (LaMondia, 2001). This symptom definitely appeared also on *N. attenuata* infected plants in Utah 2011, where on the abaxial leaf side many dark spots resembling fungal mycelium including sporulation sites could be seen. Based on that knowledge about typical disease symptoms caused by *Fusarium sp*. and *Alternaria sp.*, it can be concluded that the infection by a single fungal pathogen isolated from diseased leaves cannot suffiently explain the observed disease symptoms of infected *N. attenuata* plants in Utah. It is known that *F. solani* which caused sudden death syndrome of soybean was found to be associated with numerous common saprophytic fungi species, especially *A. alternata*,a species from which it remained unclear for that study whether it can really cause disease lesions or was just a secondary invader (Melgar *et al*., 1994). Therefore it is possible that the observed disease symptoms of *N. attenuata* plants in Utah desert were also caused by a combination of at least that two fungal pathogen genera, *Alternaria* and *Fusarium.* This hypothesis is supported by a recent disease outbreak happening in Utah in summer 2012, wiping out almost an entire *N. attenuata* planting intended to be used for field experiments. ITS-sequencing of fungal isolates gained from diseased plants (roots and leaves) resulted again mainly in *Alternaria* and *Fusarium* species (unpublished results) emphasizing to the relevance of those for *N. attenuata´*s fitness in nature.

Since fungi of the *Alternaria* genus are well-known for their variability in pathogenicity as well as morphological characters (Slavov *et al*, 2004), it is necessary to identify *Alternaria* strains to species level to be able to assess their biological characteristics useful for research. In order to identify the 12 *Alternaria* sp. Utah isolates to species level, different approaches of classification were used. Firstly, 12 *Alternari*a isolates were identified by ITS plus LSU sequencing. Unfortunately, those *Alternaria* species turned out to belong to a group of small-spored *Alternaria sp.* which include *A. alternata, A. mali, A. tenuissima* and *A. longipes*, which made species assignment on molecular genetic level quite impossible. It has been reported that ITS sequencing does not resolve those closely related *Alternaria* species are highly similar in their genome (Kusaba and Tsuge, 1995; Pryor and Gilbertson, 2000). Recently, several papers from Andersen *et al*., 2001 and 2008 have been published with a new method of classification of small-spored *Alternaria sp*. based on chemical separation. Following suggestions from those publications, the secondary metabolite profiles of all *Alternaria sp*. Utah isolates were analyzed together with *Fusarium solani* Utah and *Fusarium brachygibbosum* Utah isolates using LC-ToF-MS. As a result, there was a strong separation into three distinct groups of *Alternaria sp*. Utah isolates. The first group contained *Alternaria sp*. Utah 4, 8 and 10. The second group contained three isolates of *Alternaria sp.* Utah 1, 11 and 12, while the third one held the *Alternaria sp*. Utah 2, 3, 5, 6 and 9. Because of missing reference species strains, metabolic profiling of the resulting *Alternaria* subgroups could not be used to further assign those subgroups to certain *Alternaria* species. Therefore, another tool of classification also based on the production of species-specific metabolites was of great value for the classification of the *Alternaria* isolates (Andersen *et al*., 2001). The presence of AOH, AME, AlxI and TeA in all *Alternaria* sp. Utah isolates restricts the classification of the *Alternaria sp.* Utah isolates to *A. alternata* and *A. tenuissima.* However, *Alternaria sp.* Utah 4 and 8 did not produce Tentoxin which is a natural cyclic tetrapeptide produced by *A. alternata* and *A. tenuissima* as reported from Santolini, 1999. The absence of tentoxin could partially be explained by a loss of the fungal ability to produce tentoxin at a certain step during fungal propagation. The analysis of *Alternaria* specific mycotoxin production reduced the number of possible species to assign to the different *Alternaria sp.* Utah isolates, however it was still not sufficient to identify them exactly up to species level. Using the classical method of *Alternaria* species identification based on morphology described by Simmons and Roberts (1993), *Alternaria sp.* Utah 10 showed the typical sporulation pattern as described for *A. alternata* with a single suberect conidiophore and an apical cluster of branching conidia chains. In combination with the previous results from metabolic profiling, it can be assumed that *Alternaria sp.* Utah 4 and 8 are also *A. alternata.*

However, the morphological characters were not sufficient to identify all 12 *Alternaria sp*. Utah isolates since many of them could not be stimulated to sporulate, maybe explainable as a negative consequence of multiple propagations. Finally, other scientists suffering from the same problems of classifying small-spored *Alternaria* species recommend to refer to all small-spored *Alternaria* species as *A. alternata* with different pathotypes, representing their host specificity (Otani and Kohmoto, 1992; Johnson *et al*., 2001; Masunaka *et al*., 2005). According to that it would be obviously the best to rename all wild tobacco-associated small-spored *Alternaria* species into *A. alternata* tobacco pathotype until further envidence at genetic or physiological level can be provided to differentiate them.

6.2 Suitable pathogens for studying of *N. attenuata* – native fungal pathogen interaction

N. attenuata plants are well- known for their defense system against herbivory gained over billion years of evolution in desert conditions where wildfire is needed for their germination (Baldwin and Morse, 1994). Historically, wildfire cycles occur at return intervals of 32 to 70 years (Pellant *et al*., 2004), therefore wild tobacco seeds may need to wait for thousands of years to germinate. After germinating, the plants face many different enemies like insect herbivores they had never experienced before. As a result, *N. attenuata* plants must have a "smarty defense system" encoded in their genome in order to keep the balance between costly defense and fitness consequences (Baldwin, 2001). This could be true for the evolved defense system of *N. attenuata* against its natural pathogenic microbial community since no microbial disease outbreak in nature was reported for this plant species so far. To be able to unravel this "smarty defense system" also regarding natural fungal enemies, suitable pathogens and experimental procedures needed to be established in order to investigate plant responses against pathogen under controlled laboratory conditions.

To identify promising candidates among the 23 Utah fungal isolates, a detached leaf assay was chosen to quickly screen them for their pathogenicity. This sort of assay was a reliable and fast screening method which allowed to get a first impression regarding the pathogenicity of the different isolates. However, responses from detached leaves are not necessarily comparable to those from intact plants, as seen so nicely for *F. brachygibbosum* Utah 4, where HR lesions occurred on detached leaves causing nearly complete tissue collapse within just a few days, but were completely lacking on intact plants. Moderate and aggressive isolates were then used to infect intact *N. attenuata* seedlings using spore suspensions of different concentrations. By comparing spore application via spraying, the

root-dip method was more reliable and effective. By taking seedlings out of their growth medium, small wounding sites were created at the plant root which could serve as "entry gate" for Utah fungi, conditions maybe also mimicking the natural conditions in which *N. attenuata* faces a variety of nematodes of the genera *Ditylenchus*, *Pratylenchus*, *Tylenchorhynchus*, *Meloidogyne* and *Aphelenchoides* that could cause wounding sites on the roots (M. Erb, M. Mcclure and R. Machado, unpublished results). In contrast, the Utah fungi were by far less effectively infected by spraying, although the spore suspension not only covered the leaf surface, but also reached the roots only a few layers of sand particles underneath the surface. However, the plants stayed intact if they were sprayed, probably the strongest difference between both tested spore application methods. The cuticle has been well characterized for its function in protecting plants against pathogen attack in other plant species (Kerstiens, 1996). Since *N. attenuata* is known for their relatively thick cuticle (Hettenhausen *et al*., 2012), it may provide an especially strong protection against pathogens. Interestingly, all the Utah fungal species, except for *F. solani,* were only able to infect young plants which were younger than 20 days. Especially the most aggressive isolate of *F. brachygibbosum* can infect only 10 days-old seedlings. Therefore it can be speculated that *N. attenuata* plants may possess an "age-related resistance" to their natural pathogenic fungal community. The susceptibility of *N. attenuata* plant to Utah fungi at young stage may be caused by their evolution, since this plant germinates only after wildfire where pathogenic fungi remaining in the soil are swept. Intestinally, the similar pattern of this age-related resistance was reported in *Nicotiana benthamiana,* a closely relative species to our plant where mature plants were resistant to the pathogen *Phytopthora infestans* whereas relatively young plants were susceptible to this pathogen. The mechanism behind it remains unclear, however it was predicted to be related to a mechanisms similar to R protein-mediated recognition of pathogens (Shibata *et al*., 2010)

Furthermore, while testing an experimental design of an infection assay and making little modifications afterwards in subsequent assays in order to optimize them, it could be confirmed that plant sensitivity at young stage is particularly big since small changes in growth conditions of seedlings prior to infection had already strong impact on the infection process. Nevertheless, *A. alternata* Utah 10 infection led consistently to the induction of SA and JA in *N. attenuata*. This study demonstrates that *A. alternata* Utah 10 is able to infect *N. attenuata* plants under laboratory conditions and induces defense reponses in this plant. Therefore it can be recommended to use for further experiments with that plant species. The infection assay worked best with 10 days-old seedlings incubated in climate a chamber (20^{o}C,

16 h light and 20°C, 8 h dark, 60% humidity) for 2 days before dipping their roots into a spore suspension of 10^5 spores/ml.

Strong effects (regarding symptoms, phytohormone and secondary metabolite levels) coming from little experimental setup changes could be also observed for *F. brachygibbosum* Utah 4. The best infection setup found in this study for *F. brachygibbosum* Utah 4 is to use 8 days-old seedlings incubated in climate chamber (20°C, 16 h light and 20°C, 8 h dark, 60% humidity) for 2 days before dipping their root into spore suspension of 10^7 spores/ml.

Besides *A. alternata* Utah 10 and *F. brachygibbosum* Utah 4, *F. solani* Utah 4 could be used also for research on plant defense response towards pathogenic fungi as long as a laboratory with S2 security level is available. A preliminary infection setup for this fungal species worked well with 10 days-old seedlings grown in glasshouse (26-28ºC under 16 h supplemental light and 8h dark) for 10 days before dipping their roots into a spore suspension of 10^5 spores/ml. However, this infection setup may need to be further optimized. A detached leaf assay in which pathogen resistance to *F. solani* Utah 4 was assessed with 99 genetically different plants from different accessions revealed that this pathogen may be an useful tool to screen for differences in plant response.

In summary, each of Utah fungal infection assays was able to provide information about individual Utah fungal species for further study of Utah fungal interaction with *N. attenuata* plants. The use of young seedlings appeared to be considerably effective since all three Utah fungal species were able cause disease symptom and alter plant response.

6.3 *F. brachygibbosum* Utah 4 - a potential screening tool to compare responses of different *N. attenuata* genotypes toward their native fungal pathogens.

Considering the observations from the fungal disease outbreak in Utah 2011 where most of the diseased plants seemed to be randomly distributed within the population and diseased plants were growing next to healthy plants, it was predicted that genetic variation between plants plays an important role to define whether plants are resistant or susceptible to the pathogen. In fact, when *F. brachygibbosum* Utah 4 was used to infect different transgenic lines it was efficient to differentiate the transgenic genotypes regarding their response towards the infection. The transgenic genotypes irgla1 and aslox3 silenced in early steps of JA biosynthesis were most susceptible to *F. brachygibbosum* Utah 4 and had the highest number of dead and diseased plants. Transgenic *N. attenuata* lines are very powerful tools to investigate the role of certain genes during fungi infection. For instance, by using gene silencing based on RNAi technique, the role of JA biosynthesis was targeted, as well as JA-Ile

perception and secondary metabolite production. This fungus species might provide also a useful tool to distinguish naturally defense-compromised plants in nature.

F. brachygibbosum Utah 4 did not differentiate Utah and Arizona WT in their responses towards the fungal infection, suggesting that UtWT and AzWT plants are quite similar in resistance traits to this pathogen. However, since they were inbred for 31 generations in case of UtWT and 21 generations in case of AzWT, some of the resistance traits might have been lost during inbreeding. The difference in their responses to the native pathogen may still be seen in native plant population. In fact, it was demonstrated when *F. solani* Utah 4 was used to screen for resistance of 99 *N. attenuata* native plants collected from 31 different accessions in Utah and Arizona States of USA. There was a huge variation in response to *F. solani* Utah 4, from hyper-resistance and resistance to susceptibility and hyper-susceptibility. Although it was tested by detached leaf assay, it partly represents the actual response of those plants to the pathogen. This result indicates the diversity in response of *N. attenuata* plants towards their native pathogens and could be used to explain the random distribution of natural diseased plants in Utah 2011 by genetic differences causing genotype-dependent alterations in pathogen susceptibility and changes in disease progression.

6.4 Unraveling molecular and chemical responses of *N. attenuata* to Utah fungi.

Besides searching for a suitable Utah fungal pathogen species useful for *N. attenuata* infection assays, the initial plant responses to these fungi at phytohormone and secondary metabolite level was also addressed. SA and JA in general are two important phytohormones which are well known for their affeciency and specificity in mediating plant resistance against fungal biotropths and necrotrophs (Thomma *et al*., 1998; McDowell and Dangl, 2000; Glazebrook, 2005; Spoel *et al*., 2007). Moreover, both signaling pathways are involved in regulating resistance response against hemibiotrophic fungi which live as biotrophs in the early stage of infection, deriving nutrients from living host cells, but then switching to a necrotrophic life style in the late stage of infection, absorbing nutrients from dead tissue (Park *et al*., 2009). In this study all three pathogen species (*F. brachygibbosum* Utah 4, *F. solani* Utah 4 and *A. alternata* Utah 10) were able to induce both, SA and JA in *N .attenuata* plants. This hints to a hemibiotrophic life style of those fungi. This is in full agreement with literature where most of all *Fusarium sp.* are reported to have a hemibiotrophic life style (Thatcher *et al*., 2009), however most of the *Alternaria sp*. are reported to possess a necrotrophic fungi. As quoted so often, there is no rule without exception, and this holds also true for *Alternaria* since some of the *Alternaria* species are reported to be hemibiotrophic, including *A. alternata*

(Duan *et al*., 2010). It could be shown that SA was induced by the Utah fungi earlier than JA. Many authors already had shown that SA increased resistance of plants to hemibiotrophic fungi due to SA mediated signaling, for instance, SA is needed for resistance of *Nicotiana benthamiana* against *Phytophthora infestans* (Shibata *et al*., 2010), and induced SA-dependent signaling increased resistance against Fusarium wilt (*F. oxysporum* f. sp. *lycopersici*) in tomato (Shcherbakova *et al*., 2011). However in this work, using ovNahG plants which accumulate less SA (Gilardoni *et al*., 2011) exhibited no significant increase in susceptibility of *N. attenuata* to *F. brachygibbosum* Utah 4. This indicated that SA does not play an essential role in *N. attenuata´*s resistance against *F. brachygibbosum* Utah 4. The induction of SA may function in cooperation with JA since they have been described to have synergistic effects (Schenk *et al*., 2000; Mur *et al*., 2006). For examples, induced systemic resistance which is induced by non-pathogenic plant growth promoting bacteria functions independently of SA, but requires the SA signal transducer NPR1 which is regulated by JA (Walters and Heil, 2007). By contrast, SA seems to play an important role in *N. attenuata* defense against *A. alternata* Utah 10 since ovNahG plants were more susceptible to *A. alternata* Utah 10 than wild type. Since *A. alternata* Utah 10 behaved as a biotroph at the early stage of infection, the mechanism behind the induction of SA is probably similar to that of other biotrophs where the SA-dependent signaling pathway controls the synthesis of low molecular weight antimicrobial proteins such as PR-1, PR-2 and PR-5 (Park *et al*., 2009). However, assessing the role of SA based solely on phenotypic observations on ovNahG plants may be inaccurate. Therefore, further investigations using different SA-deficient lines will be needed in order to drive more valuable conclusions on the role of SA in *N. attenuata* defense against its natural pathogens.

Although the plant defense responses mediated by JA are often necessary for resistance against necrotrophic pathogen (Glazebrook, 2005), this study showed that JA and JA mediated signaling pathways are responsible also for the resistance to hemibiotrophic pathogens like *F. brachygibbosum* Utah 4. Transgenic lines such as aslox3 which are compromised in the accumulation of JA were highly susceptible to *F. brachygibbosum* Utah 4 compared to Utah wildtype, indicating the important role of JA in resistance against this fungus. Similar results were found in defense responses of *Arabidopsis thaliana* to *Sclerotinia sclerotiorum* where aos mutants, also deficient in biosynthesis of JA, were highly susceptible to this pathogen (Stotz *et al*., 2011). Interestingly irgla1 plants were more susceptible to the pathogen than aslox3. Silencing *GLA1* did not change biosynthesis of C6 volatiles by the hydroperoxide lyase (HPL) pathway but it suppresses DVE accumulation during the early stage of infection by *Phytophthora parasitica* (var. nicotianae) (Bonaventure *et al*., 2011). In

another study on oxylipin production in *Nicotiana tobacum* in response to *Phytophthora parasitica* (var. nicotianae), it was shown that DVE are essential for full pathogen resistance (Fammartino *et al*., 2007). From this, it was hypothesized that DVE might play a role in resistance of *N. attenuata* against *F. brachygibbosum* Utah 4. Indeed an induction of 9-DVE-18:2 at 2 dai with *A. alternata* Utah 10 could be observed.

Transgenic *N. attenuata* plants impaired in JA-perception (ircoi1), but not JA-biosynthesis were shown to be highly resistant to *F. brachygibbosum* Utah 4. In other words, COI1 is needed for successful colonization of this fungus in *N. attenuata* plants. Similar findings were already reported for *F. oxysporum* in *Arabidopsis thaliana* (Thatcher *et al*., 2009). Thatcher *et al*., 2009 also suggested a model in which COI1-dependent signaling could explain the highly resistant phenotype of the coi1 mutant. COI1, after perceiving the signal JA-Ile would induce non-defensive JA-mediated responses which promote pathogen-induced symptom development, for example senescence program.

Interestingly, C_{12} derivatives from HPL pathway including traumatic acid, OH traumatin and/or traumatin were shown to increase in infected plants with *A. alternata* Utah 10 and *F. brachygibbosum* Utah 4. Recently, Kallenbach *et al*., 2011 reported that some of those molecules may work as signals during the responses of *N. attenuata* to wounding and herbivory. It would be interesting to investigate the function of those compounds in pathogen defense signaling.

Among the JA-inducible or herbivore-inducible secondary metabolites of *N. attenuata* plants including alkaloids (e.g. nicotine), phenolics (e.g. cryptochlorogenic acid) and phenylpropanoid-polyamine conjugates (PPCs) (e.g. caffeoyl putrescine [CP] and dicaffeoylspermidine [DCS]) (Keinanen *et al*., 2001; Kessler and Baldwin, 2002; Kaur *et al*., 2010), some are known to influence herbivore performance. For instance, nicotine, caffeoyl putrescine and dicaffeoylspermidine were found to inhibit growth of both generalist and specialist herbivores of *N. attenuata* (Krischik *et al*., 1991; Steppuhn *et al*., 2004; Kaur *et al*., 2010). This study showed that nicotine was induced after *F. brachygibbosum* Utah 4 infection and since irpmt plant (low nicotine) were more susceptible to *F. brachygibbosum* Utah 4 than Utah wildtype plant, it can be concluded that nicotine plays a role in resistance against Utah fungi. The role of PPCs and particular DCS in defense against Utah fungi was tested using irmyb8 plants that completely lack of CP and DCS (Kaur *et al*., 2010). As a result, irmyb8 plants were more susceptible to *F. brachygibbosum* Utah 4 than wildtype, suggesting that dicaffeoylspermidine may confer resistance of *N. attenuata* against its natural fungal enemies. Support for this hypothesis comes from reports about hypersensitive reactions and resistance in plant-fungi interactions related to the accumulation of some phenylamides such as

conjugates of cinnamic and p-coumaric acids with tyramine and octopamine that appeared in potato tubers early after inoculation with an avirulent isolate of *Phytophthora infestans* (Clarke, 1982). Moreover, they appeared also to be involved in the resistance to fungal pathogens based on the formation of cell wall appositions, barrier structures which arrest the attempted fungal penetration into host plant tissues (Edreva *et al*., 2007). While the full role of MYB8-regulated phenylamides in defense against herbivores in *N. attenuata* was obtained (Onkokesung *et al*., 2012), this work is the first step to explore its role in defense against pathogens in this plant species.

Besides JA-inducible secondary metabolites in *N. attenuata*, the flavonoids rutin and chlorogenic acid, which are not induced by jasmonates or insect herbivory (Keinanen *et al*., 2001; Kessler *et al*., 2004; Wu *et al*., 2008) were found to be induced respectively by *F. brachygibbosum* Utah 4 and all three Utah fungal species. Rutin was reported to may have antiherbivore function and chlorogenic acid can be an important resistance factor against thrips (Krischik *et al*., 1991; Hoffmann-Campo *et al*., 2001; Leiss *et al*., 2009). It is tempting to speculate that rutin and chlorogenic acid may play a role in pathogen defense response of native tobacco too.

6.5 Interaction of *N. attenuata* with *P. indica* and two pathogen species (*F. brachygibbosum* or *A. alternata*).

A qPCR approach was used in order to quantify the two fungal pathogens *F. brachygibbosum* and *A. alternata* in infected *N. attenuata* plants pre-inoculated with the mutualistic fungus *P. indica*. However, this attempt failed. Even though the primers used for amplification of *Alternaria sp.* Utah 10 were previously published to be useful for *A. alternata* quantification in another solanaceous plant (tomato) where their species-specificity was shown by comparing amplicons in PCR with DNA from 90 fungal strains, including all major tomato pathogens (Schuhegger *et al*., 2006), and an initial PCR result was in full agreement with this, the primers clearly resulted in unspecific amplification during qPCR since Ct values of the pathogen were almost at the same level as the reference gene (translation elongation factor 1a from *N. attenuata*), even in uninfected control samples. Quantification of *F. brachygibbosum* also failed because the Ct threshold was either never reached during 40 cycles or Ct values were in the range of "noise" (random unspecific amplification, Ct values between 35 and 40). Optimization of the qPCR conditions (different primers and changes in the qPCR program like e.g. annealing temperature) could be used to follow this quantification attempt in future. The quantification of *P. indica* revealed strong

benefit for this species if *F. brachygibbosum* or *A. alternata* were used to infect *N. attenuata*. There are several potential explanations available for this phenomenon: It is known that *P. indica* and *S. vermifera,* another species from the *Sebacinaceae* family closely related to *P. indica*, can reduce the plant´s ability to induce JA and JA-inducible responses like trypsine proteinase inhibitors typically elicited by insect herbivory (Barazani *et al*., 2007), but *P. indica* inoculation can lead also to changes in the levels of various other phytohormones like auxins, cytokinins, ABA and ethylene (Vadassery *et al*., 2008), some of which might be also relevant to *N. attenuata´*s interaction with *F. brachygibbosum* or *A. alternata*. Both pathogens were demonstrated to cause the opposite effect compared to *P. indica* at least on JA levels (induction of JA), thereby maybe changing the plants phytohormone balance necessary to restrict *P. indica* colonization to certain regions of the root (Khatabi *et al*., 2012). If this growth restriction control by the plant host is not effective any more, *P. indica* could colonize also other plant parts, resulting in increased *P. indica* biomass per plant. Another possible could be provided by the fact that all infected seedlings developed disease symptoms really rapidly and quite severe during this experiment compared to earlier infection assays. Since *P. indica* is also able to grow saprophytically (Varma *et al*., 1999; Deshmukh and Kogel, 2007), maybe it benefits from nutrients provided by dead host cells killed by *F. brachygibbosum* or *A. alternata* in the heavily diseased plants.

It remains to be important to quantify colonization by *F. brachygibbosum* Utah 4 or *A. alternata* Utah 10 after pre-inoculation with *P. indica*, not only to study tripartite interactions, but also to find an effective method to prevent field experimental disasters like the one described above by using seedlings pre-inoculated either with *P. indica* or other potential biocontrol agents in the future. Also the efficiency of different fungicide treatments could be tested using the individual pathogens or a combination of them.

7 Conclusion

A natural fungal disease outbreak opened a door for us to enter an entirely new world of discovering *N. attenuata´*s interaction with microbial pathogens. Successful isolation and identification of fungal species from diseased leaves could be used to establish infection assays under laboratory conditions, providing a tool to further investigate the plant's response to pathogens useful to distinguish naturally defense-compromised *N. attenuata* plants in the wild populations.

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10 Supplemental

Supplemental Table 1: List of stably transformed lines of *Nicotiana attenuata* used in this study

Lines harbor sense (*ov*), antisense (*as*) or inverted repeats (*ir*) constructs

Line	Accession	Vector/Cross	Selectable marker	Gene (s)	Reference
irGLA1	Ut	pSOL8GLA1	hygromycin	glycerolipase A1	(Bonaventure *et al*., 2011)
asLOX3	Ut	pNATLOX	nourseothricin	lipoxygenase 3	(Halitschke and Baldwin, 2003)
irCOI1	Ut	pSOL3COI1	hygromycin	coronatine insensitive 1	(Paschold *et al*., 2007)
irJAR4/6	Ut	irJAR4 x irJAR6	hygromycin	Jasmonate resistant 4 and jasmonate resistant 6	(Wang *et al*., 2008)
irPMT	Ut	pRESC5PMT	hygromycin	putrescine N-methyltransferase	(Zavala *et al*., 2004)
irMYB8	Ut	pSOL8MYB8	hygromycin	MYB transcription factor 8	(Kaur *et al*., 2010a)
ovNahG	Ut	pSOL1NAHG1	hygromycin	salycilate hydroxylase	(Gilardoni *et al*., 2011)

Supplemental Table 2: Accession list of *Nicotiana attenuata* wild type plants collected from Utah and Arizona, USA.

Accession	Localtion description	No. of plants	Accession	Localtion description	No. of plants
1-2A	Bulk lower field cost side Apex mine 7/4/96	4	3-1F	Motoqua burn	5
1-2C	BSUCS 7/6/96	5	5-2A	PUP bulk 1990	2
19-1C	Wash above Motoqua burn 1/8/2000	1	3-1C	Kearsley Ranch AZ 8/28/95	2
19-1D	Bird ranch Beaver dam wash 12/13/2008	6	3-1G	Benton X-road California 8/26/95	2
3-1B	DI-dump bulk 8/22/95	2	3-1D	Before shot up CAN site 8/29/95	1
10-A	F1 from Apex mine seed batch 1998	3	22-5A-5J	Lytle wash (mills wash) 4/9/2003 5A-5J	5
22-coll. 2	Merry UT/AZ coll.2006 coll. 2 Highway 91	9	22-coll. 3	Merry UT/AZ coll.2006 coll. 3 Highway 91	1
4-1H+I+J+K+L	Indiv. Plants DI ranch upper canyon by pump	3	22-6A-6U	Site 6 Rt96 burn middle by dam 2003 burn 9/4/2004 6A-6U	1
4-2A+B+C	Indiv. Plants little mormon house Motoqua road	1	21-1 F,G,H	Indiv. Plant road between C'B camp and Motoqua 9/2/2004 1A-1T	3
21-3A-3J	Gold strike rd. Bend pop 3A-3J burned 2003 coll. 2004	5	21-1 I,J	Indiv. Plant road between C'B camp and Motoqua 9/2/2004 1A-1T	1
21-4A-4T	Pahcoon Flat burn 4A-4T 2004	3	21-1 N,K,T	Indiv. Plant road between C'B camp and Motoqua 9/2/2004 1A-1T	5
22-40 A,B	Merry UT/AZ coll.2006 coll. 4 Tule desert road	7	21-1A-1E	Indiv. Plant road between C'B camp and Motoqua 9/2/2004 1A-1T	1
23-site2/A-E	Individual plant collections 6/30/2009 site 2 cowboy camp intersection	4	21-2A-2T	DI burn 9/2/2004 20indiv. Plants 2A-2T burned 2003	4
23-site3/A-U	Individual plant collections 6/30/2009 site 3 wash pop	1	22-42 ABC	Merry UT/AZ coll.2006 coll. 4 Tule desert road	5
23-site4/A-Q	Individual plant collections 6/30/2009 site 4 water-inlet upper canyon	7	22-43 A,B,C	Merry UT/AZ coll.2006 coll. 4 Tule desert road	5
			23site1/1-20	Individual plant collections 6/30/2009 site 1 gate pop	2

Supplemental Table 3. Detached leaf assay with *F. solani* Utah 4 using different *N. attenuata* accessions

		Acquired resistance		**Innate resistance**	**Product**	Ranking
Plant Number	Accession	Hypersensitive Reaction (HR) lesion (cm)	HR lesion (% of max)	Chlorosis (%)	(acquired resistance x innate resistance)	R+: Hyper-resistance R: Resistance S: susceptible S+: Hyper-susceptible
1	22-42 ABC	0.00	0	5	0	R+
2	22-40 A,B	0.00	0	5	0	R+
3	4-1H+I+J+K+L	0.00	0	10	0	R+
4	21-3A-3J	0.00	0	10	0	R+
5	21-3A-3J	0.00	0	15	0	R+
6	0-Utah	0.00	0	20	0	R+
7	3-1F	0.00	0	25	0	R+
8	3-1G	0.00	0	30	0	R+
9	3-1B	0.00	0	30	0	R+
10	21-1 N,K,T	0.00	0	50	0	R+
11	21-3A-3J	0.70	11	5	54	R+
12	22-42 ABC	1.75	27	5	135	R+
13	3-1G	2.91	45	5	226	R+
14	23-site4/A-Q	1.59	25	10	247	R+
15	3-1F	0.53	8	30	247	R+
16	23-site4/A-Q	3.51	54	5	272	R
17	4-1 H+I+K+L	0.88	14	20	273	R
18	22-5A-5J	0.90	14	30	419	R
19	23-site4/A-Q	2.82	44	10	436	R
20	10-A	1.32	20	25	512	R
21	21-3A-3J	3.31	51	10	512	R
22	3-1B	1.18	18	30	549	R
23	22-40 A,B	3.69	57	10	572	R
24	22-43 A,B,C	1.23	19	30	572	R
25	1-2A	0.76	12	60	707	R
26	22-43 A,B,C	1.57	24	30	728	R
27	21-1 F,G,H	1.10	17	45	767	Indifferent
28	21-4A-4T	1.68	26	30	781	Indifferent
29	21-2A-2T	1.01	16	50	783	Indifferent
30	21-2A-2T	0.96	15	55	814	Indifferent
31	23-site2/A-E	1.79	28	30	833	Indifferent
32	22-43 A,B,C	2.40	37	25	928	Indifferent
33	21-3A-3J	4.15	64	15	964	Indifferent
34	21-1A-1E	2.08	32	30	965	Indifferent
35	5-2A	6.45	100	10	1000	Indifferent
36	23-site2/A-E	1.71	26	40	1057	Indifferent
37	3-1F	1.60	25	45	1116	Indifferent
38	21-2A-2T	1.20	19	60	1116	Indifferent
39	23-site2/A-E	1.87	29	40	1160	Indifferent

Table 3 Continued

Plant Number	Accession	Acquired resistance: Hypersensitive Reaction (HR) lesion (cm)	Acquired resistance: HR lesion (% of max)	Innate resistance: Chlorosis (%)	Product: (acquired resistance x innate resistance)	R+: Hyper-resistance R: Resistance S: susceptible S+: Hyper-susceptible
40	22-43 A,B,C	1.54	24	50	1190	Indifferent
41	3-1G	3.98	62	20	1234	Indifferent
42	22-40 A,B	4.09	63	20	1268	Indifferent
43	21-1A-1E	1.67	26	50	1295	Indifferent
44	22-43 A,B,C	2.88	45	30	1337	Indifferent
45	10-A	1.83	28	50	1419	Indifferent
46	22-5A-5J	1.92	30	50	1488	Indifferent
47	22-5A-5J	2.15	33	50	1663	Indifferent
48	4-1H+I+J+K+L	3.64	56	30	1693	Indifferent
49	22-coll. 2	2.22	34	50	1717	Indifferent
50	3-1F	1.62	25	70	1753	Indifferent
51	21-4A-4T	2.55	40	45	1779	Indifferent
52	22-coll. 3	2.94	46	40	1823	Indifferent
53	3-1F	2.51	39	50	1942	Indifferent
54	22-coll. 2	2.11	33	60	1963	Indifferent
55	23-site2/A-E	2.57	40	50	1988	Indifferent
56	21-4A-4T	2.58	40	50	1996	Indifferent
57	21-2A-2T	2.71	42	50	2101	Indifferent
58	10-A	1.97	31	70	2138	Indifferent
59	22-coll. 2	2.91	45	50	2256	Indifferent
60	22-5A-5J	3.08	48	50	2384	Indifferent
61	3-1C	1.82	28	90	2533	S
62	22-6A-6U	2.95	46	60	2744	S
63	1-2C	2.25	35	80	2791	S
64	21-1 F,G,H	3.91	61	50	3027	S
65	5-2A	2.49	39	80	3082	S
66	1-2A	2.12	33	95	3115	S
67	21-1 I,J	3.66	57	60	3400	S
68	1-2C	2.31	36	95	3402	S
69	19-1D	2.76	43	80	3423	S
70	23-site4/A-Q	2.36	37	100	3651	S
71	21-1 N,K,T	3.15	49	75	3663	S
72	23-site4/A-Q	3.98	62	60	3698	S
73	1-2A	3.48	54	70	3771	S
74	23-site3/A-U	3.12	48	80	3870	S
75	1-2C	2.65	41	95	3903	S
76	4-2A+B+C	4.23	66	60	3930	S
77	21-1 N,K-T	3.31	51	80	4105	S+
78	22-coll. 2	3.32	51	80	4112	S+
79	19-1D	2.80	43	95	4124	S+
80	23-site4/A-Q	2.83	44	95	4161	S+

Table 3 Continued

Plant Number	Accession	Acquired resistance: Hypersensitive Reaction (HR) lesion (cm)	Acquired resistance: HR lesion (% of max)	Innate resistance: Chlorosis (%)	Product: (acquired resistance x innate resistance)	R+: Hyper-resistance R: Resistance S: susceptible S+: Hyper-susceptible
81	22-42 ABC	4.07	63	70	4412	S+
82	19-1D	4.23	66	70	4591	S+
83	19-1D	3.78	59	80	4682	S+
84	1-2C	4.06	63	80	5036	S+
85	19-1D	3.28	51	100	5085	S+
86	19-1D	3.54	55	95	5207	S+
87	22-coll. 2	4.30	67	80	5333	S+
88	22-42 ABC	3.64	56	95	5354	S+
89	3-1C	3.47	54	100	5372	S+
90	19-1C	3.47	54	100	5380	S+
91	22-coll. 2	3.50	54	100	5426	S+
92	22-coll. 2	3.69	57	95	5428	S+
93	22-5A-5J	4.58	71	80	5674	S+
94	23site1/1-20	4.12	64	100	6380	S+
95	21-1A-1E	4.39	68	95	6459	S+
96	3-1D	4.24	66	100	6574	S+
97	21-1 F,G,H	5.04	78	100	7806	S+
98	23site1/1-20	5.71	88	100	8845	S+
99	1-2C	5.77	89	100	8946	S+

Supplemental Table 4. List of ions with VIP values larger or equal to 1

Ref No.: Reference number for individual ion
RT: Retention time (seconds)
VIP: Variable Importance in the Projection
unk: unkown metabolite

Ref No.	m/z	RT(s)	Identity	VIP	Rank
485	541.0642	245.3484	unk	1.99	1
269	314.0544	373.5744	unk	1.97	2
390	413.0877	302.7855	unk	1.96	3
484	540.0676	245.2348	unk	1.95	4
254	294.0222	225.1589	unk	1.95	5
482	539.0629	245.2348	unk	1.95	6
431	457.1106	302.6637	unk	1.93	7
496	582.1381	305.6047	unk	1.93	8
277	317.0456	373.8519	unk	1.93	9
228	277.032	225.2468	unk	1.93	10
322	350.0753	245.1397	unk	1.92	11
448	473.0769	245.1461	unk	1.92	12
233	278.0702	201.2245	unk	1.92	13
268	313.0503	373.8624	unk	1.92	14
544	860.8477	19.24495	unk	1.91	15
308	345.0401	303.7417	unk	1.91	16
447	472.0789	244.8781	unk	1.90	17
464	490.0895	231.5819	unk	1.90	18
463	489.0853	231.5239	unk	1.90	19
444	471.0755	244.8781	unk	1.90	20
261	301.0508	373.9224	unk	1.89	21
471	517.0803	244.7539	unk	1.89	22
506	607.052	245.4348	unk	1.88	23
473	518.084	244.7579	unk	1.88	24
315	347.0565	400.0645	unk	1.87	25
363	385.0333	268.0092	unk	1.86	26
450	474.0776	244.9308	unk	1.85	27
42	151.0408	193.5766	unk	1.84	28
493	561.0506	245.763	unk	1.84	29
358	379.0481	248.4585	unk	1.82	30
123	208.0327	239.7747	unk	1.82	31
118	206.9722	3.257829	unk	1.81	32
160	229.0113	239.8903	unk	1.81	33
554	1064.808	16.47182	unk	1.81	34
318	348.0595	400.0764	unk	1.80	35
459	485.0467	369.3354	unk	1.79	36
260	301.05	267.9499	unk	1.79	37
224	275.1026	148.8547	unk	1.79	38

Table 4 continued

Ref No.: Reference number for individual ion

RT: Retention time (seconds)

VIP: Variable Importance in the Projection

unk: unkown metabolite

Ref No.	m/z	RT(s)	Identity	VIP	Rank
129	210.0493	225.0896	unk	1.76	39
61	164.0437	239.7747	unk	1.76	40
125	209.0448	225.1533	unk	1.76	41
483	539.0632	283.5883	unk	1.75	42
425	453.0626	272.5698	unk	1.75	43
409	418.063	369.3223	unk	1.73	44
119	207.0297	239.7563	unk	1.73	45
489	548.1932	314.4269	unk	1.73	46
492	553.2989	374.3674	unk	1.71	47
494	564.1866	306.8086	unk	1.71	48
347	365.0664	269.4167	unk	1.70	49
406	417.0598	369.331	unk	1.70	50
57	163.0404	192.3071	unk	1.69	51
117	206.972	19.82624	unk	1.69	52
196	253.0744	214.4998	unk	1.69	53
449	473.0774	283.5883	unk	1.67	54
481	537.0485	315.8822	unk	1.67	55
58	163.0404	239.6844	unk	1.67	56
445	471.0756	283.5906	unk	1.66	57
350	367.082	307.7933	unk	1.66	58
446	472.0788	283.622	unk	1.66	59
41	151.0414	223.0274	unk	1.62	60
379	399.0491	373.873	unk	1.61	61
551	943.1577	244.5281	unk	1.61	62
419	436.1031	315.4567	unk	1.61	63
60	164.0435	192.3071	unk	1.61	64
443	470.0637	315.8455	unk	1.61	65
293	329.046	316.1846	unk	1.60	66
274	316.0668	372.9043	unk	1.60	67
400	416.0472	376.3616	unk	1.58	68
295	331.0611	369.4357	unk	1.58	69
441	469.0601	315.8792	unk	1.58	70
324	350.0754	369.331	unk	1.57	71
321	349.0719	369.331	unk	1.57	72
307	343.1404	235.221	unk	1.57	73
421	441.0804	225.2468	unk	1.57	74
114	200.0934	263.3417	unk	1.56	75
326	351.0774	369.2894	unk	1.56	76
460	487.0622	316.1347	unk	1.56	77

Table 4 continued

Ref No.: Reference number for individual ion

RT: Retention time (seconds)

VIP: Variable Importance in the Projection

unk: unkown metabolite

Ref No.	m/z	RT(s)	Identity	VIP	Rank
264	305.0807	345.1346	unk	1.56	78
528	725.8765	19.48087	unk	1.56	79
442	469.0597	237.6398	unk	1.56	80
455	483.0311	376.3716	unk	1.55	81
256	295.1539	282.9722	unk	1.54	82
368	387.0636	315.1186	unk	1.53	83
271	315.0655	372.915	unk	1.52	84
395	415.0437	376.3751	unk	1.51	85
100	192.0656	222.8375	unk	1.51	86
1	107.0518	222.7046	unk	1.49	87
113	200.0924	204.3134	unk	1.49	88
50	158.0828	122.7518	unk	1.49	89
381	401.0637	372.915	unk	1.49	90
116	206.0814	227.5772	unk	1.48	91
331	352.0912	315.3494	unk	1.48	92
335	353.0936	315.3905	unk	1.48	93
411	419.0754	315.4567	unk	1.47	94
328	351.0874	315.2044	unk	1.47	95
422	441.1185	315.3573	unk	1.46	96
298	333.077	315.2729	unk	1.46	97
314	347.0563	376.3751	unk	1.46	98
152	222.0761	163.9561	unk	1.45	99
539	793.8636	5.255176	unk	1.45	100
320	348.0601	376.3716	unk	1.44	101
142	215.0821	230.3747	unk	1.44	102
259	297.0706	197.3493	unk	1.44	103
49	158.0826	205.2483	unk	1.44	104
263	303.0934	307.1123	unk	1.44	105
232	278.066	213.2633	unk	1.43	106
378	398.0967	315.3739	unk	1.43	107
257	295.2274	466.153	unk	1.43	108
294	330.0488	376.3751	unk	1.42	109
82	181.0506	163.0076	unk	1.42	110
364	385.9392	19.29141	unk	1.41	111
291	329.0458	376.3751	unk	1.41	112
265	309.1234	225.0951	unk	1.41	113
377	397.0938	315.3739	unk	1.40	114
76	179.0355	222.4229	unk	1.39	115
56	163.0403	314.6582	unk	1.39	116

Table 4 continued

Ref No.: Reference number for individual ion

RT: Retention time (seconds)

VIP: Variable Importance in the Projection

unk: unkown metabolite

Ref No.	m/z	RT(s)	Identity	VIP	Rank
380	399.0989	315.3494	unk	1.39	117
221	274.068	227.7393	unk	1.39	118
252	291.0977	157.4892	unk	1.39	119
8	115.0423	119.4074	unk	1.38	120
59	163.0405	218.17	unk	1.38	121
311	346.0441	373.8605	unk	1.38	122
70	173.0101	89.84678	unk	1.37	123
339	361.2013	387.978	unk	1.37	124
52	159.031	89.81631	unk	1.36	125
225	275.1025	208.9063	unk	1.36	126
440	467.0435	342.4257	unk	1.36	127
204	258.0967	242.6631	unk	1.35	128
17	128.0372	89.41855	unk	1.35	129
507	607.1251	322.6693	unk	1.35	130
272	315.0661	315.4567	unk	1.35	131
296	332.9789	389.9535	unk	1.34	132
360	383.0781	262.4953	unk	1.34	133
420	441.0651	269.0478	unk	1.32	134
375	395.0776	321.6721	unk	1.32	135
522	658.8905	4.374796	unk	1.31	136
227	276.1032	208.9588	unk	1.30	137
13	121.0315	221.9564	unk	1.29	138
310	345.0414	374.2819	unk	1.26	139
461	487.2185	229.4437	unk	1.26	140
553	996.8207	18.05427	unk	1.26	141
466	499.0706	237.5801	unk	1.26	142
156	226.9655	56.1439	unk	1.25	143
346	365.0669	320.2475	unk	1.25	144
4	112.9876	59.66853	unk	1.25	145
126	209.0465	175.1614	unk	1.25	146
188	250.9638	4.275168	unk	1.24	147
498	588.8972	4.936103	unk	1.24	148
172	241.117	177.6767	unk	1.24	149
64	166.0598	180.4075	unk	1.23	150
99	192.0228	89.76386	unk	1.23	151
102	193.0507	150.0597	unk	1.21	152
430	455.1015	61.85582	unk	1.21	153
97	191.0191	89.98155	unk	1.21	154
51	158.9794	53.92981	unk	1.20	155

Table 4 continued

Ref No.: Reference number for individual ion

RT: Retention time (seconds)

VIP: Variable Importance in the Projection

unk: unkown metabolite

Ref No.	m/z	RT(s)	Identity	VIP	Rank
72	174.9563	54.68357	unk	1.20	156
130	210.0759	199.3702	unk	1.20	157
131	210.1121	349.6658	unk	1.20	158
209	261.0877	182.2156	unk	1.19	159
106	194.0811	160.0377	unk	1.18	160
137	212.0919	183.4331	unk	1.17	161
534	758.8626	4.539864	unk	1.16	162
359	379.084	69.58518	unk	1.15	163
63	165.0559	180.5724	unk	1.15	164
356	377.0856	69.95046	unk	1.15	165
136	212.0916	218.714	unk	1.14	166
69	172.0979	210.7248	unk	1.13	167
415	431.1726	374.402	unk	1.13	168
369	387.1148	62.69253	unk	1.11	169
135	212.092	197.3764	unk	1.11	170
413	429.1904	387.9913	unk	1.11	171
479	522.9152	18.76308	unk	1.10	172
337	361.0347	329.0724	unk	1.10	173
371	388.1182	62.80953	unk	1.10	174
9	117.0216	94.80794	unk	1.10	175
365	385.9388	5.117292	unk	1.10	176
426	453.9261	19.26026	unk	1.10	177
141	214.1077	242.1296	unk	1.10	178
348	366.0701	320.6237	unk	1.09	179
170	239.0664	471.9263	unk	1.08	180
183	249.1123	366.7697	unk	1.08	181
343	363.1793	306.7614	unk	1.08	182
75	177.0557	298.7096	unk	1.08	183
245	288.9367	53.61356	unk	1.07	184
11	119.0509	239.211	unk	1.07	185
211	265.072	232.7906	unk	1.06	186
336	357.0595	300.7845	unk	1.05	187
147	221.0447	298.7032	unk	1.05	188
215	269.0446	312.6513	unk	1.04	189
270	314.1138	218.7186	unk	1.03	190
175	245.0926	236.7164	unk	1.03	191
474	520.9099	4.974971	unk	1.03	192
12	119.0512	192.1742	unk	1.03	193
177	247.0218	222.4907	unk	1.02	194

Table 4 continued

Ref No.: Reference number for individual ion

RT: Retention time (seconds)

VIP: Variable Importance in the Projection

unk: unkown metabolite

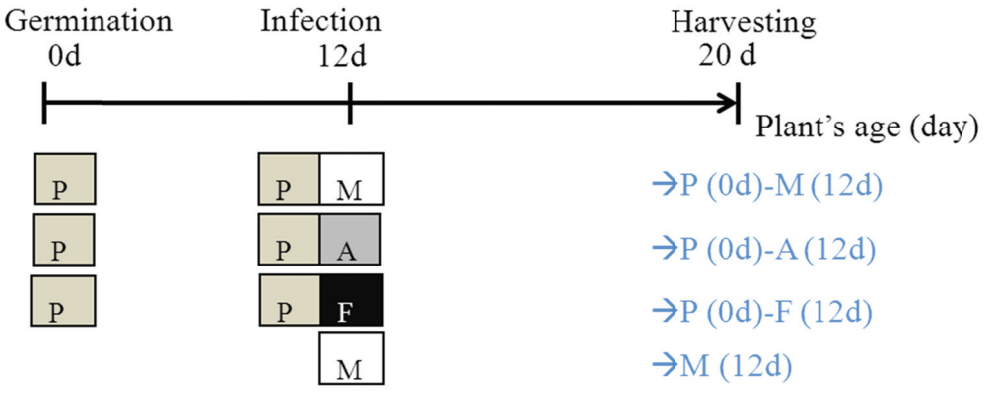
Ref No.	m/z	RT(s)	Identity	VIP	Rank
150	222.0483	298.6671	unk	1.02	195
115	204.1238	20.8344	unk	1.01	196
148	221.0803	205.072	unk	1.01	197
43	152.0442	223.0436	unk	1.00	198
138	213.0375	104.4044	unk	1.00	199
146	219.027	223.2078	unk	1.00	200
151	222.0764	199.3664	unk	1.00	201
168	235.021	138.9498	unk	1.00	202
305	342.2002	306.7614	unk	1.00	203
306	343.0912	209.0437	unk	1.00	204
351	369.1924	345.0696	unk	1.00	205
465	490.1721	381.4113	unk	1.00	206
208	260.1088	167.2545	unk	1.00	207

Supplemental Table 5. The production of important mycotoxins by *Alternaria* species

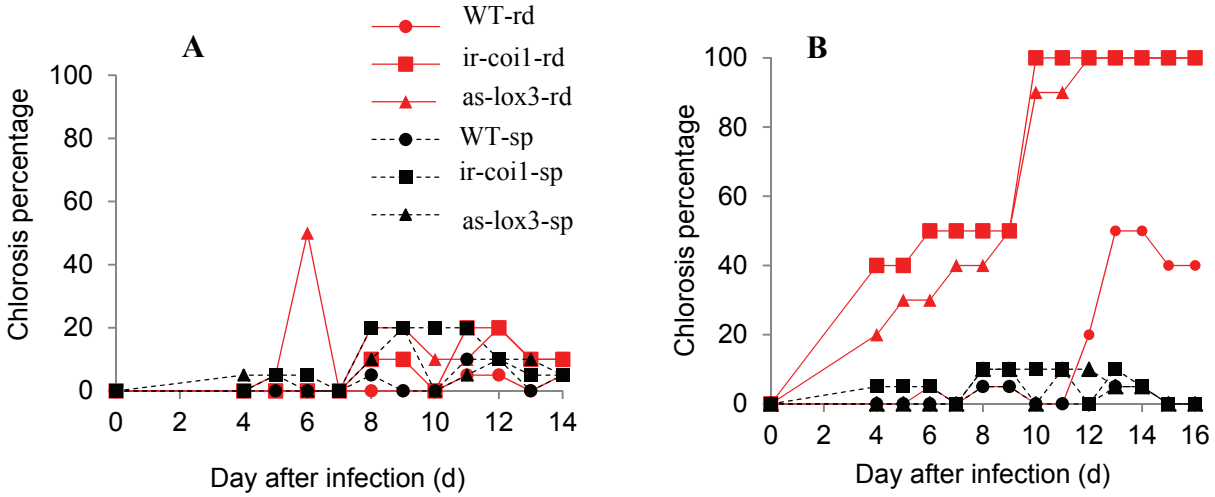
Species	Mycotoxin	References
A. alternata	AOH, AME, Alt, Alx I, II, III, TeA, Ten	Bilgrami *et al*., 1994; Andersen *et al*., 2001; Andersen *et al*., 2005; Solfrizzo *et al*., 2005; Andersen *et al*., 2008; Ostry, 2008
A. tenuissima	AOH, AME, Alt, Alx I, III, TeA, Ten	Davis et al., 1977; Bilgrami et al., 1994; Andersen *et al*., 2002; Ostry, 2008
A. mali	Alx I, II, III, TeA	Kinoshita, *et al*., 1972; Ostry, 2008
A. longipes	AME, Alx I, TeA	Mikami *et al*., 1971; Andersent *et al*., 2001; Andersen *et al*., 2005

Supplemental Figure 1: Multiple infections setup of *N. attenuata* plants with a mutualistic fungus and Utah pathogenic fungi

Following the time line of plant's age: at germination date (0d), *N. attenuata* seeds were placed on *P. indica* pre-inoculated GB5 plates (P); when plants were 12 days- old, they all were either infected with Utah fungi (*A. alternata* Utah 10 (A) and/or *F. brachygibbosum* Utah 4 (F)) or treated with MgSO**4** (M) as a mock treatment. After 8 days, all plants were harvested and samples list is shown in the Figure.



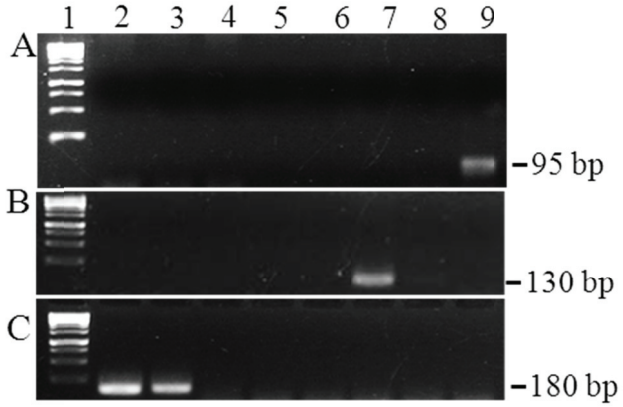
Supplemental Figure 2: Chlorosis percentage of *N. attenuata* plants after infection with *A. alternata* Utah 10 and *F. brachygibbosum* Utah 5



A. Percentage of chlorosis of 10 days-old *N. attenuata* plants which were infected with *F. brachygibbosum* Utah 4 using concentration of 10^7spores/ml. **B**. Percentage of chlorosis of 10 days-old *N. attenuata* plants which were infected with *A. alternata* Utah 10 using concentration of 10^5spores /ml. WT, as-lox3 and ir-coi1 plants are marked with round, triangle and square marker respectively. Method of rootdip (rd-red-solid line) and spraying (sp-black-dashed line) was used.

Supplemental Figure 3: Confirmation of specific primers for quantitative PCR

Gel electrophoresis of PCR-amplified products using (A) *A. alternata* specific primers (alt-F3,R1.1), (B) *F. brachygibbosum* Utah 10 specific primers (F. bra-F,R) and (C) *N. attenuata* specific primers (NaEF1a-F,R). Lane 1, 1kb DNA ladder; lane 2 and 3, *N. attenuata*; lane 4, *Phytophthora parasitica* var. *nicotianae*; lane 5, *Piriformospora indica*; lane 6, *Sebacina vermifera*; lane 7, *Fusarium brachygibbossum* Utah 4; lane 8, *Fusarium solani* Utah 4; lane 9, *Alternaria alternata* Utah 10.



Supplemental Data 1: ITS+LSU sequences of 23 Utah fungal isolates

>Utah fungal isolate No. 1 (*F. brachygibbosum* Utah 1)

5'AACAAGGTACTCCCGTCTCGGTGAACCAGCGGAGGGGATCATTACCGAGTTTACAACTCCCAAA
CCCCTGTGAACATACCTTTATGTTGCCTCGGCGGATCAGCCCGCGCCCCGTAAAACGGGACGGCCC
GCCGCAGGAACCACAAAACTCTGATTTTAGTGTAACTTCTGAGTCTAAAAAACAAATAAATCAAAA
CTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGT
GAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGG
GCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCCCGGGTTTGGTGTTGGGGATCGGGCTGTAC
TCCAGCCCGGCCCCGAAATCTAGTGGCGGTCTCGCTGCAGCCTCCATTGCGTAGTAGCTAACACCT
CGCAACTGGAACGCGGCGCGGCCAAGCCGTTAAACCCCCAACTTCTGAATGTTGACCTCGGATCAG
GTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCC
CTAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCTCTCGGGCCCGAGTTGTAA
TTTGTAGAGGATGCTTTTGATGCGGTGCCTTCCGAGTTCCCTGGAACGGGACGCCATAGAGGGTGA
GAGCCCCGTCTGGTTGGATGCCAAATCTCTGTAAAGCTCCTTCGACGAGTCGAGTAGTTTGGGAAT
GCTGCTCTAAATGGGAGGATTATGTCTTCTAAAGCTAAATACCGGCCAGAGACCGATAGCGCACAA
GTAGAGTGATCGAAAGATGAAAAGCACTTTGAAAAGAGAGTTAAAAAGTACGTGAAATTGTTGAA
AGGGAAGCGTTTATGACCAGACTTGGGCTTGGTTAATCATCTGGGGTTCTCCCCAGTGCACTTTTCC
AGTCCAGGCCAGCATCAGTTTTCGCCGGGGGATAAAGGCTTCGGGAATGTGGCTCCCTCCGGGGAG
TGTTATAGCCCGTTGCGTAATACCCTGGCGGGGACTGAGGTTCGCGCTTCTGCAAGGGAGTCGGCG
TAATTCTACAACAT3'

>Utah fungal isolate No. 2 (*F. brachygibbosum* Utah 2)

5'GGTACTCCCGTCTCGGTGAACCAGCGGAGGGATCATTACCGAGTTTACAACTCCCAAACCCCTGT
GAACATACCTTTATGTTGCCTCGGCGGATCAGCCCGCGCCCCGTAAAACGGGACGGCCCGCCGCAG
GAACCACAAAACTCTGATTTTAGTGTAACTTCTGAGTCTAAAAAACAAATAAATCAAAACTTTCAA
CAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGC
AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCC
TGTTCGAGCGTCATTTCAACCCTCAAGCCCCCGGGTTTGGTGTTGGGGATCGGGCTGTACTCCAGCC
CGGCCCCGAAATCTAGTGGCGGTCTCGCTGCAGCCTCCATTGCGTAGTAGCTAACACCTCGCAACT
GGAACGCGGCGCGGCCAAGCCGTTAAACCCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGA
ATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCCTAGTA
ACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCTCTCGGGCCCGAGTTGTAATTTGTA
GAGGATGCTTTTGATGCGGTGCCTTCCGAGTTCCCTGGAACGGGACGCCATAGAGGGTGAGAGCCC
CGTCTGGTTGGATGCCAAATCTCTGTAAAGCTCCTTCGACGAGTCGAGTAGTTTGGGAAATGCTGC
TCTAAAATGGGAGGTATATGTCTTCTAAAGCTAAATACCGGCCAGAGACCGATAGCGCACAAGTA
GAGTGATCGAAAGATGAAAAGCACTTTGAAAAGAGAGTTAAAAAGTACGTGAAATTGTTGAAAGG
GAAGCGTTTATGACCAGACTTGGGCTTGGTTAATCATCTGGGGTTCTCCCCAGTGCACTTTTCCAGT
CCAGGCCAGCATCAGTTTTCGCCGGGGGATAAAGGCTTCGGGAATGTGGCTCCCTCCGGGGAGTGT
TATAGCCCGTTGCGTAATACCCTGGCGGGGACTGAGGTTCGCGCTTCTGCAAGGGATGCTCGCGTA
ATTCTACAAC3'

>Utah fungal isolate No. 3 (*F. brachygibbosum* Utah 3)

5'CGTCTCGGTGTAACCAGCGGAGGGGCTTCATTACCGAGTTTACATCTCCCAAACCCCTGTGAACA
TACCTTTATGTTGCCTCGGCGGATCAGCCCGCGCCCCGTAAAACGGGACGGCCCGCCGCAGGAACC
ACAAAACTCTGATTTTAGTGTAACTTCTGAGTCTAAAAAACAAATAAATCAAAACTTTCAACAACG
GATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAAT
TCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCG
AGCGTCATTTCAACCCTCAAGCCCCCGGGTTTGGTGTTGGGGATCGGGCTGTACTCCAGCCCGGCC
CCGAAATCTAGTGGCGGTCTCGCTGCAGCCTCCATTGCGTAGTAGCTAACACCTCGCAACTGGAAC
GCGGCGCGGCCAAGCCGTTAAACCCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCC

GCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCCTAGTAACGGCG
AGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCTCTCGGGCCCGAGTTGTAATTTGTAGAGGAT
GCTTTTGATGCGGTGCCTTCCGAGTTCCCTGGAACGGGACGCCATAGAGGGTGAGAGCCCCGTCTG
GTTGGATGCCAAATCTCTGTAAAGCTCCTTCGACGAGTCGAGTAGTTTGGGAATGCTGCTCTAAAT
GGGAGGTATATGTCTTCTAAAGCTAAATACCGGCCAGAGACCGATAGCGCACAAGTAGAGTGATC
GAAAGATGAAAAGCACTTTGAAAAGAGAGTTAAAAAGTACGTGAAATTGTTGAAAGGGAAGCGTT
TATGACCAGACTTGGGCTTGGTTAATCATCTGGGGTTCTCCCCAGTGCACTTTTCCAGTCCAGGCCA
GCATCAGTTTTCGCCGGGGGATAAAGGCTTCGGGAATGTGGCTCCCTCCGGGGAGTGTTATAGCCC
GTTGCGTAATAACCCTGGCGGGGACTGAGGGTTCGGCGGCTTCTGCAAGGGATGTCTCG3’

>Utah fungal isolate No. 4 (*F. brachygibbosum* Utah 4)

5’AACAAGGTACTCCCGTCTCGGTGAACCAGCGGAGGGGATCATTACCGAGTTTACAACTCCCAAA
CCCCTGTGAACATACCTTTATGTTGCCTCGGCGGATCAGCCCGCGCCCCGTAAAACGGGACGGCCC
GCCGCAGGAACCACAAAACTCTGATTTTAGTGTAACTTCTGAGTCTAAAAAACAAATAAATCAAAA
CTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGT
GAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGG
GCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCCCGGGTTTGGTGTTGGGGATCGGGCTGTAC
TCCAGCCCGGCCCCGAAATCTAGTGGCGGTCTCGCTGCAGCCTCCATTGCGTAGTAGCTAACACCT
CGCAACTGGAACGCGGCGCGGCCAAGCCGTTAAACCCCCAACTTCTGAATGTTGACCTCGGATCAG
GTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCC
CTAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCTCTCGGGCCCGAGTTGTAA
TTTGTAGAGGATGCTTTTGATGCGGTGCCTTCCGAGTTCCCTGGAACGGGACGCCATAGAGGGTGA
GAGCCCCGTCTGGTTGGATGCCAAATCTCTGTAAGCTCCTTCGACGAGTCGAGTAGTTTGGGAATG
CTGCTCTAAATGGGAGGTATATGTCTTCTAAAGCTAAATACCGGCCAGAGACCGATAGCGCACAAG
TAGAGTGATCGAAAGATGAAAAGCACTTTGAAAAGAGAGTTAAAAAGTACGTGAAATTGTTGAAA
GGGAAGCGTTTATGACCAGACTTGGGCTTGGTTAATCATCTGGGGTTCTCCCCAGTGCACTTTTCCA
GTCCAGGCCAGCATCAGTTTTCGCCGGGGGATAAAGGCTTCGGGAATGTGGCTCCCTCCGGGGAGT
GTTATAGCCCGTTGCGTAATACCCTGGCGGGGACTGAGGTTCGCGCTTCTGCAAGGATGCTCGCGT
AATTCTACAAC3’

>Utah fungal isolate No. 5 (*F. solani* Utah 1)

5’AGGCGGGGGGGCATGCATCTACCGAGTTATACAACTCATTCAACCCTGTGAACATACCTATAACG
TTGCCTCGGCGGGAACAGACGGCCCCGTAACACGGGCCGCCCCCGCCAGAGGACCCCCTAACTCTG
TTTCTATAATGTTTCTTCCGAGAAAGAAGCAAATAAATTAAAACTTTCAACAGCGGATCTCTTGGCT
CTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCA
TCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTAC
AACCCTCAGGCCCCCGGGCCTGGCGTTGGGGATCGGCGGAAGCCCCCTGCGGGCACAACGCCGTC
CCCCAAATACAGTGGCGGTCCCGCCGCAGCTTCCATTGCGTAGTAGCTAACACCTCGCAACTGGAG
AGCGGCGCGGCCACGCCGTAAAACACCCAACTTCTGAATGTTGACCTCGAATCAGGTAGGAATACC
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GAGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCTCTCGGGCCCGAGTTGTAATTTGTAGAGGA
TGCTTTTGGTGAGGTGCCTTCCGAGTTCCCTGGAACGGGACGCCATAGAGGGTGAGAGCCCCGTCT
GGTTGGACACCGATCCTCTGTAAAGCTCCTTCGACGAGTCGAGTAGTTTGGGAATGCTGCTCTAAA
TGGGAGGTATATGTCTTCTAAAGCTAAATACCGGCCAGAGACCGATAGCGCACAAGTAGAGTGAT
CGAAAGATGAAAAGAACTTTGAAAAGAGAGTTAAAAAGTACGTGAAATTGTTGAAAGGGAAGCGC
TTGTGACCAGACTTGGGCTTGGTTGATCATCCGGGGTTCTCCCCGGTGCACTCTTCCGGCTCAGGCC
AGCATCAGTTCGCCCTGGGGGATAAAGGCTTCGGGAATGTGGCTCTCTCCGGGGAGTGTTATAGCC
CGCTGCGTAATACCCTGTGGCGGACTGAGGTTCGCGGCATTCGGCAAGGGATGTCTTGGGTATATT
3’

>Utah fungal isolate No. 6 (*F. solani* Utah 2)

5’GTTCCGTTGGTGAACCAGGCGGGGGGCTCATCTACCGAGTTATACAACTCATCAACCCTGTGAAC
ATACCTATAACGTTGCCTCGGCGGGAACAGACGGCCCCGTAACACGGGCCGCCCCCGCCAGAGGA
CCCCCTAACTCTGTTTCTATAATGTTTCTTCTGAGAAAAGAAGCAAATAAATTAAAACTTTCAACAA
CGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGA
ATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTT
CGAGCGTCATTACAACCCTCAGGCCCCCGGGCCTGGCGTTGGGGATCGGCGGAAGCCCCCTGCGGG
CACAACGCCGTCCCCCAAATACAGTGGCGGTCCCGCCGCAGCTTCCATTGCGTAGTAGCTAACACC
TCGCAACTGGAGAGCGGCGCGGCCACGCCGTAAAACACCCAACTTCTGAATGTTGACCTCGAATCA
GGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGC
CCCAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCTCTCGGGCCCGAGTTGTA
ATTTGTAGAGGATGCTTTTGGTGAGGTGCCTTCCGAGTTCCCTGGAACGGGACGCCATAGAGGGTG
AGAGCCCCGTCTGGTTGGACACCGATCCTCTGTAAAGCTCCTTCGACGAGTCGAGTAGTTTGGGAA
TGCTGCTCTAAATGGGAGGTATATGTCTTCTAAAGCTAAATACCGGCCAGAGACCGATAGCGCACA
AGTAGAGTGATCGAAAGATGAAAAGAACTTTGAAAAGAGAGTTAAAAAGTACGTGAAATTGTTGA
AAGGGAAGCGCTTGTGACCAGACTTGGGCTTGGTTGATCATCCGGGGTTCTCCCCGGTGCACTCTT
CCGGCTCAGGCCAGCATCAGTTCGCCCTGGGGGATAAAGGCTTCGGGAATGTGGCTCTCTCCGGGG
AGTGTTATAGCCCGCTGCGTAATACCCTGTGGCGGAC3’

>Utah fungal isolate No. 7 (*F. solani* Utah 3)

5’AGGCGGGGGGGCATCATCTACCGAGTTATACAACTCATTCAACCCTGTGAACATACCTATAACGT
TGCCTCGGCGGGAACAGACGGCCCCGTAACACGGGCCGCCCCCGCCAGAGGACCCCCTAACTCTGT
TTCTATAATGTTTCTTCTGAGCAAAGAAGCAAATAAATTAAAACTTTCAACAACGGATCTCTTGGCT
CTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCA
TCGAAGTCTTTGAACGCACATTGCGCCCGCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTAC
AACCCTCAGGCCCCCGGGCCTGGCGTTGGGAATCGGCGGAAGCCCCCTGCGGGCACAACGCCGTC
CCCCAAATACAGTGGCGGTCCCGCCGCAGCTTCCATTGCGTAGTAGCTAACACCTCGCAACTGGAG
AGCGGCGCGGCCACGCCGTAAAACACCCAACTCTGAATGTGACCTCGAATCAGGTAGGAATACCG
CTGAACTTAAGCATTCAATAAGCGGAGGAAAAGAAACCAACAGGGATGCCCCAGTAACGGCGAGT
GAACGGCCACAGCTCAAATTTGAAATCTGGCTCTCGGGCCCGAGTTGTAATTTGTAGAGGATGCTT
TGGTGAGGTGCCTCCGAGTTCCCTGGAACGGGACGCCATAGAGGGTGAGAGCCCCGTCTGGTTGGA
CACCGATCCTCTGTAAAGCTCCTTCGACGAGTCGAGTAGTTTGGGAATGCTGCTCTAAATGGGAGG
TATATGTCTTCTAAAGCTAAATACCGGCCAGAGACCGATAGCGCACAAGTAGAGTGATCGAAAGA
TGAAAAGAACTTTGAAAAGAGAGTTAAAAAGTACGTGAAATTGTTGAAAGGGAAGCGCTTGTGAC
CAGACTTGGGCTTGGTTGATCATCCGGGGTTCTCCCCGGTGCACTCTTCCGGCTCAGGCCAGCATCA
GTTCGCCCTGGGGGATAAAGGCTTCGGGAATGTGGCTCTCTCCGGGGAGTGTTATAGCCCGCTGCG
TAATACCCTGTGGCGGACTGAGGTTCGCGCATTCGCAAGGATGCTGGTGTAAT3’

>Utah fungal isolate No. 8 (*F. solani* Utah 4)

5’GATCCAGGCGGGGGGGCTGCATTACCGAGTTATACAACTCATCAACCCTGTGAACATACCTATAA
CGTTGCCTCGGCGGGAACAGACGGCCCCGTAACACGGGCCGCCCCCGCCAGAGGACCCCCTAACT
CTGTTTCTATAATGTTTCTTCCGAGAAAAGACGCAAATAAATTAAAACTTTCAACAACGGATCTCTT
GGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGA
ATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCGAGCGTCA
TTACAACCCTCAGGCCCCCGGGCCTGGCGTTGGGATCGGCGGAAGCCCCCTGCGGGCACAACGCCG
TCCCCCAAATACAGTGGCGGTCCCGCCGCAGCTTCCATTGCGTAGTAGCTAACACCTCGCAACTGG
AGAGCGGCGCGGCCACGCCGTAAAACACCCAACTTCTGAATGTTGACCTCGAATCAGGTAGGAAT
ACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCCCAGTAAC
GGCGAGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCTCTCGGGCCCGAGTTGTAATTTGTAGA
GGATGCTTTTGGTGAGGTGCCTTCCGAGTTCCCTGGAACGGGACGCCATAGAGGGTGAGAGCCCCG

TCTGGTTGGACACCGATCCTCTGTAAAGCTCCTTCGACGAGTCGAGTAGTTTGGGAATGCTGCTCTA
AATGGGAGGTATATGTCTTCTAAAGCTAAATACCGGCCAGAGACCGATAGCGCACAAGTAGAGTG
ATCGAAAGATGAAAAGAACTTTGAAAAGAGAGTTAAAAAGTACGTGAAATTGTTGAAAGGGAAGC
GCTTGTGACCAGACTTGGGCTTGGTTGATCATCCGGGGTTCTCCCCGGTGCACTCTTCCGGCTCAGG
CCAGCATCAGTTCGCCCTGGGGGATAAAGGCTTCGGGAATGTGGCTCTCTCCGGGGAGTGTTATAG
CCCGCTGCGTAATACCCTGTGGCGGACTGAGGTTCGCGCATTCGCAAGGGATGCTTG3’

>Utah fungal isolate No. 9 (*F. solani* Utah 5)

5’GTCTCGGTGAACCAGGCGGGGGGGCTCATCTACCGAGTTATACAACTCATTCAACCCTGTGAACA
TACCTATAACGTTGCCTCGGCGGGAACAGACGGCCCCGTAACACGGGCCGCCCCCGCCAGAGGAC
CCCCTAACTCTGTTTCTATAATGTTTCTTCCGAGAAAAGAAGCAAATAAATTAAAACTTTCAACAAC
GGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGA
ATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTT
CGAGCGTCATTACAACCCTCAGGCCCCCGGGGCCTGGCGTTGGGGATCGGCGGAAGCCCCCTGCGG
GCACAACGCCGTCCCCCAAATACAGTGGCGGTCCCGCCGCAGCTTCCATTGCGTAGTAGCTAACAC
CTCGCAACTGGAGAGCGGCGCGGCCACGCCGTAAAACACCCAACTTCTGAATGTTGACCTCGAATC
AGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTG
CCCCAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCTCTCGGGCCCGAGTTGT
AATTTGTAGAGGATGCTTTTGGTGAGGTGCCTTCCGAGTTCCCTGGAACGGGACGCCATAGAGGGT
GAGAGCCCCGTCTGGTTGGACACCGATCCTCTGTAAAGCTCCTTCGACGAGTCGAGTAGTTTGGGA
ATGCTGCTCTAAATGGGAGGTATATGTCTTCTAAAGCTAAATACCGGCCAGAGACCGATAGCGCAC
AAGTAGAGTGATCGAAAGATGAAAAGAACTTTGAAAAGAGAGTTAAAAAGTACGTGAAATTGTTG
AAAGGGAAGCGCTTGTGACCAGACTTGGGCTTGGTTGATCATCCGGGGTTCTCCCCGGTGCACTCT
TCCGGCTCAGGCCAGCATCAGTTCGCCCTGGGGGATAAAGGCTTCGGGAATGTGGCTCTCTCCGGG
GAGTGTTATAGCCCGCTGCGTAATACCCTGTGGCGGACTGAGGTTCGCGCATTCGCAAGGATGCTT
GGTG3’

>Utah fungal isolate No. 10 (*F. solani* Utah 6)

5’GACAAGGTGTTCCGTCTCGGTGATCCAGGCGGGGGGGCTCATTACCGAGTTATACAACTCATTCA
ACCCTGTGAACATACCTATAACGTTGCCTCGGCGGGAACAGACGGCCCCGTAACACGGGCCGCCCC
CGCCAGAGGACCCCCTAACTCTGTTTCTATAATGTTTCTTCCGAGAAAAGAAGCAAATAAATTAAA
ACTTTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATG
TGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCG
GGCATGCCTGTTCGAGCGTCATTACAACCCTCAGGCCCCCGGGCCTGGCGTTGGGGATCGGCGGAA
GCCCCCTGCGGGCACAACGCCGTCCCCCAAATACAGTGGCGGTCCCGCCGCAGCTTCCATTGCGTA
GTAGCTAACACCTCGCAACTGGAGAGCGGCGCGGCCACGCCGTAAAACACCCAACTTCTGAATGTT
GACCTCGAATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACC
AACAGGGATTGCCCCAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCTCTCG
GGCCCGAGTTGTAATTTGTAGAGGATGCTTTTGGTGAGGTGCCTTCCGAGTTCCCTGGAACGGGAC
GCCATAGAGGGTGAGAGCCCCGTCTGGTTGGACACCGATCCTCTGTAAAGCTCCTTCGACGAGTCG
AGTAGTTTGGGAATGCTGCTCTAAATGGGAGGTATATGTCTTCTAAAGCTAAATACCGGCCAGAGA
CCGATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGAACTTTGAAAAGAGAGTTAAAAAGTAC
GTGAAATTGTTGAAAGGGAAGCGCTTGTGACCAGACTTGGGCTTGGTTGATCATCCGGGGTTCTCC
CCGGTGCACTCTTCCGGCTCAGGCCAGCATCAGTTCGCCCTGGGGGATAAAGGCTTCGGGAATGTG
GCTCTCTCCGGGGAGTGTTATAGCCCGCTGCGTAATACCCTGTGGCGGACTGAGGTTCGCGCATTC
GCAAGGGATGCT3’

>Utah fungal isolate No. 11 (*Fusarium. sp.* Utah 1)

5’AGCCGGGGCGGTATCATTTACTCGAGTTTACAACTCCCAAACCCCTGTGAACATACCTTACTGTT
GCCTCGGCGGATCAGCCCGCTCCCGGTAAAACGGGACGGCCCGCAGAGGACCCCAAACTCTGTTTC
TATATGTTACTTCCGAGACAAGACATAAATAAATCACATCTTTCAACAGCGGATCTCTTGGTTCTGG
CATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGA
ATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGGCATGCCTGTTCGAGCGTCATTTCAAC
CCTCAAGCCCTCGGGTTTTGGTGTTGGGGATCGGCGAGCCTTTCTGGCAAGCCGGCCCCGAAATCT
AGTGGCGGTCTCGCTGCAGCCTCCATTGCGTAGTAGTAAAACCCTCGCAACTGGAACGCGGCGCGG
CCAAGCCGTTAAACCCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTT
AAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCCTAGTAACGGCGAGTGAAGC
GGCAACAGCTCAAATTTGAAATCTGGCTCTCGGGCCCGAGTTGTAATTTGTAGAGGATGCTTTTGA
TGCGGTGCCTTCCGAGTTCCCTGGAACGGGACGCCATAGAGGGTGAGAGCCCCGTCTGGTTGGATG
CCAAATCTCTGTAAAGCTCCTTCAACGAGTCGAGTAGTTTGGGAATGCTGCTCTAAATGGGAGGTA
TATGTCTTCTAAAGCTAAATACCGGCCAGAGACCGATAGCGCACAAGTAGAGTGATCGAAAGATG
AAAAGCACTTTGAAAAGAGAGTTAAAAAGTACGTGAAATTGTTGAAAGGGAAGCGTTTATGACCA
GACTTGGACTTGGTTAATCATCTGGGGTTCTCCCCAGTGCACTTTTCCAGTTCAGGCCAGCATCAGT
TTTCCCCGGGGGATAAAGGCGGCGGGAATGTGGCTCTCTTCGGGGAGTGTTATAGCCCGCCGTGTA
ATACCCTGGGGGGGACTGAGGTTCGCGCATCTGCAAGGGA3’

>Utah fungal isolate No. 12 (*Alternaria sp.* Utah 1)

5’CGTCAAGGTACTCCGTAGGTGAACCTGCGGGGGGGATCATCTACACAAATATGAAGGCGGGCTG
GAACCTCTCGGGGTTACAGCCTTGCTGAATTATTCACCCTTGTCTTTTGCGTACTTCTTGCTTCCTTG
GCGGGCTCGCCCACCACTAGGACAAACATAAACCTTTTGTAATTGCAATCAGCGTCAGTAACAAAT
TAATAATTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
GATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTG
GTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTC
TTGTCTCTAGCTTTGCTGGAGACTCGCCTTAAAGTAATTGGCAGCCGGCCTACTGGTTTCGGAGCGC
AGCACAAGTCGCACTCTCTATCAGCAAAGGTCTAGCATCCATTAAGCCTTTTTTTCAACTTTTGACC
TCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACA
GGGATTGCCCTAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCTCTTTTAGAG
TCCGAGTTGTAATTTGCAGAGGGCGCTTTGGCTTTGGCAGCGGTCCAAGTTCCTTGGAACAGGACG
TCACAGAGGGTGAGAATCCCCGTACGTGGTCCCTGGCTATTGCCGTGTAAAGCCCCCTTCGACGAG
TCGAGTTGTTTGGGAATGCAGCTCTAAATGGGAGGTACATTTCTTCTAAAGCTAAATATTGGCCAG
AGACCGATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGGAAAGAGAGTCAAACA
GCACGTGAAATTGTTGAAAGGGAAGCGCTTGCAGCCAGACTTGCTTACAGTTGCTCATCCGGGTTT
CTACCCGGTGCACTCTTCTGTAGGCAGGCCAGCATCAGTTTGGGCGGTAGGATAAAGGTCTCTGTC
ACGTACCTCCTTTCGGGGAGGCCTTATAGGGGAGACGACATACTACCAGCCTGGACTGAGGTCCGC
GCATCTGCTAGGAGCTGCGTAATGCTAACTTC3’

>Utah fungal isolate No. 13 (*Alternaria sp.* Utah 2)

5’GGAACACGGGCCAGTGATCCTGCGGGGGGGTCATTACACAAATATGAAGGCGGGCTGGAACCTC
TCGGGGTTACAGCCTTGCGGAATTATTCACCCTTGTCTTTTGCGTACTTCTTGCTTCCTTGGCGGACT
CGCCCACCACTAGGACAAACATAAACCTTTTGTAATTGCAATCAGCGTCAGTAACAAATTAATAAT
TACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGT
AGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCA
AAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTCTTGTCTCT

AGCTTTGCTGGAGACTCGCCTTAAAGTAATTGGCAGCCGGCCTACTGGTTTCGGAGCGCAGCACAA
AGTCGCACTCTCTATCAGCAAAGGTCTAGCATCCATTAAGCCTTTTTTTCAACTTTTGACCTCGGAT
CAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATT
GCCCTAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCTCTTTTAGAGTCCGAG
TTGTAATTTGCAGAGGGCGCTTTGGCTTTGGCAGCGGTCCAAGTTCCTTGGAACAGGACGTCACAG
AGGGTGAGAATCCCGTACGTGGTCGCTGGCTATTGCCGTGTAAAGCCCCTTCGACGAGTCGAGTTG
TTTGGGAATGCAGCTCTAAATGGGAGGTACATTTCTTCTAAAGCTAAATATTGGCCAGAGACCGAT
AGCGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGGAAAGAGAGTCAAACAGCACGTGA
AATTGTTGAAAGGGAAGCGCTTGCAGCCAGACTTGCTTACAGTTGCTCATCCGGGTTTCTACCCGG
TGCACTCTTCTGTAGGCAGGCCAGCATCAGTTTGGGCGGTAGGATAAAGGTCTCTGTCACGTACCT
CCTTTCGGGGAGGCCTTATAGGGGAGACGACATACTACCAGCCTGGACTGAGGTCCGCGCA3’

>Utah fungal isolate No. 14 (*Alternaria sp.* Utah 3)

5’CCTGCGGGGGGATCATTACACAAATATGAAGGCGGGCTGGAACCTCTCGGGGTTACAGCCTTGC
GGAATTATTCACCCTTGTCTTTTGCGTACTTCTTGCTTCCTTGGCGGGCTCGCCCACCACTAGGACA
AACATAAACCTTTTGTAATTGCAATCAGCGTCAGTAACAAATTAATAATTACAACTTTCAACAACG
GATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAAT
TCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCG
AGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTCTTGTCTCTAGCTTTGCTGGAGACTC
GCCTTAAAGTAATTGGCAGCCGGCCTACTGGTTTCGGAGCGCAGCACAAGTCGCACTCTCTATCAG
CAAAGGTCTAGCATCCATTAAGCCTTTTTTTCAACTTTTGACCTCGGATCAGGTAGGGATACCCGCT
GAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCCTAGTAACGGCGAGT
GAAGCGGCAACAGCTCAAATTTGAAATCTGGCTCTTTTAGAGTCCGAGTTGTAATTTGCAGAGGGC
GCTTTGGCTTTGGCAGCGGTCCAAGTTCCTTGGAACAGGACGTCACAGAGGGTGAGAATCCCGTAC
GTGGTCGCTGGCTATTGCCGTGTAAAGCCCCTTCGACGAGTCGAGTTGTTTGGGAATGCAGCTCTA
AATGGGAGGTACATTTCTTCTAAAGCTAAATATTGGCCAGAGACCGATAGCGCACAAGTAGAGTG
ATCGAAAGATGAAAAGCACTTTGGAAAGAGAGTCAAACAGCACGTGAAATTGTTGAAAGGGAAGC
GCTTGCAGCCAGACTTGCTTACAGTTGCTCATCCGGGTTTCTACCCGGTGCACTCTTCTGTAGGCAG
GCCAGCATCAGTTTGGGCGGTAGGATAAAGGTCTCTGTCACGTACCTCCTTTCGGGGAGGCCTTAT
AGGGGAGACGACATACTACCAGCCTGGACTGAGG3’

>Utah fungal isolate No. 15 (*Alternaria sp.* Utah 4)

5’AGTGCGGAACAAGGTACTCCGTAGGTGAACCTGCGGAGGGATCATTACACAAATATGAAGGCGG
GCTGGAACCTCTCGGGGTTACAGCCTTGCGGAATTATTCACCCTTGTCTTTTGCGTACTTCTTGTTTC
CTTGGTGGGTTCGCCCACCACTAGGACAAACATAAACCTTTTGTAATTGCAATCAGCGTCAGTAAC
AAATTAATAATTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAA
ATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCC
TTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGG
CGTCTTGTCTCTAGCTTTGCTGGAGACTCGCCTTAAAGTAATTGGCAGCCGGCCTACTGGTTTCGGA
GCGCAGCACAAGTCGCACTCTCTATCAGCAAAGGTCTAGCATCCATTAAGCCTTTTTTTCAACTTTT
GACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACC
AACAGGGATTGCCCTAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCTCTTTT
AGAGTCCGAGTTGTAATTTGCAGAGGGCGCTTTGGCTTTGGCAGCGGTCCAAGTTCCTTGGAACAG
GACGTCACAGAGGGGTGAGAATCCCGTACGTGGTCGCTGGCTATTGCCGTGTAAAGCCCCTTCGAC
GAGTCGAGTTGTTTGGGAATGCAGCTCTAAATGGGAGGTACATTTCTTCTAAAGCTAAATATTGGC
CAGAGACCGATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGGAAAGAGAGTCAA
ACAGCACGTGAAATTGTTGAAAGGGAAGCGCTTGCAGCCAGACTTGCTTACAGTTGCTCATCCGGG
TTTCTACCCGGTGCACTCTTCTGTAGGCAGGCCAGCATCAGTTTGGGCGGTAGGATAAAGGTCTCT
GTCACGTACCTCCTTTCGGGGAGGCCTTATAGGGGAGACGACATACTACCAGCCTGACTGAGGTCC
GCGCTCTGCTAGGAGCTGCGTAAGCTAACGT3’

>Utah fungal isolate No. 16 (*Alternaria sp.* Utah 5)

5’CAAGGAACACGGGCCGTGAACCTGCGGGGGGGTCATTACACAAATATGAAGGCGGGCTGGAACC
TCTCGGGGTTACAGCCTTGCGGAATTATTCACCCTTGTCTTTTGCGTACTTCTTGCTTCCTTGGGGGG
CTCGCCCACCACTAGGACAAACATAAACCTTTTGTAATTGCAATCAGCGTCAGTAACAAATTAATA
ATTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAA
GTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTC
CAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTCTTGTCT
CTAGCTTTGCTGGAGACTCGCCTTAAAGTAATTGGCAGCCGGCCTACTGGTTTCGGAGCGCAGCAC
AAGTCGCACTCTCTATCAGCAAAGGTCTAGCATCCATTAAGCCTTTTTTTCAACTTTTGACCTCGGA
TCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGAT
TGCCCTAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCTCTTTTAGAGTCCGA
GTTGTAATTTGCAGAGGGCGCTTTGGCTTTGGCAGCGGTCCAAGTTCCTTGGAACAGGACGTCACA
GAGGGTGAGAATCCCGTACGTGGTCGCTGGCTATTGCCGTGTAAAGCCCCTTCGACGAGTCGAGTT
GTTTGGGAATGCAGCTCTAAATGGGAGGTACATTTCTTCTAAAGCTAAATATTGGCCAGAGACCGA
TAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGGAAAGAGAGTCAAACAGCACGTGA
AATTGTTGAAAGGGAAGCGCTTGCAGCCAGACTTGCTTACAGTTGCTCATCCGGGTTTCTACCCGG
TGCACTCTTCTGTAGGCAGGCCAGCATCAGTTTGGGCGGTAGGATAAAGGTCTCTGTCACGTACCT
CCTTTCGGGGAGGCCTTATAGGGGAGACGACATACTACCAGCCTGGACTGAGG3’

>Utah fungal isolate No. 17 (*Alternaria sp.* Utah 6)

5’AGTCGACAAGGTCTCCGTAGGGTGAACCTGCGGAGGGATCATTACACAAATATGAAGGCGGGCT
GGAACCTCTCGGGGTTACAGCCTTGCTGAATTATTCACCCTTGTCTTTTGCGTACTTCTTGTTTCCTT
GGTGGGTTCGCCCACCACTAGGACAAACATAAACCTTTTGTAATTGCAATCAGCGTCAGTAACAAA
TTAATAATTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG
CGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTT
GGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGT
CTTGTCTCTAGCTTTGCTGGAGACTCGCCTTAAAGTAATTGGCAGCCGGCCTACTGGTTTCGGAGCG
CAGCACAAGTCGCACTCTCTATCAGCAAAGGTCTAGCATCCATTAAGCCTTTTTTTCAACTTTTGAC
CTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA3’

>Utah fungal isolate No. 18 (*Alternaria sp.* Utah 7)

5’AGTGCGACAAGGTACTCCGTAGGGTGAACCTGCGGGGGGATCATTACACAAATATGAAGGCGGG
CTGGAACCTCTCGGGGTTACAGCCTTGCTGAATTATTCACCCTTGTCTTTTGCGTACTTCTTGTTTCC
TTGGTGGGTTCGCCCACCACTAGGACAAACATAAACCTTTTGTAATTGCAATCAGCGTCAGTAACA
AATTAATAATTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA
TGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT
TTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGC
GTCTTGTCTCTAGCTTTGCTGGAGACTCGCCTTAAAGTAATTGGCAGCCGGCCTACTGGTTTCGGAG
CGCAGCACAAGTCGCACTCTCTATCAGCAAAGGTCTAGCATCCATTAAGCCTTTTTTTCAACTTTTG
ACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCA
ACAGGGATTGCCCTAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCTCTTTTA
GAGTCCCGAGTTGTAATTTGCAGAGGGCGCTTTGGCTTTGGCAGCGGTCCAAGTTCCTTGGAACAG
GACGTCACAGAGGGTGAGAATCCCGTACGTGGTCCCTGGCTATTGCCGTGTAAAGCCCCTTCGACG
AGTCGAGTTGTTTGGGAATGCAGCTCTAAATGGGAGGTACATTTCTTCTAAAGCTAAATATTGGCC
AGAGACCGATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGGAAAGAGAGTCAAA
CAGCACGTGAAATTGTTGAAAGGGAAGCGCTTGCAGCCAGACTTGCTTACAGTTGCTCATCCGGGT
TTCTACCCGGTGCACTCTTCTGTAGGCAGGCCAGCATCAGTTTGGGCGGTAGGATAAAGGTCTCTG
TCACGTACCTCCTTTCGGGGAGGCCTTATAGGGGAGACGACATACTACCAGCCTGGACTGAGGTCC
GCGCATCTGCTAGGATGCTGGCGTAATGGCTTAA3’

>Utah fungal isolate No. 19 (*Alternaria sp.* Utah 8)

5’AGGAACACGGGCCATTGAACTCTGCGGGGGGATCATTACACAATATGAAAGCGGGCTGGATACT
CTGTAGTAGTGCATTGCTTTACGGCGTGCGCATGGGTGGAGAGCCTACCCTTGGTGAATTATTCAC
GCGGTGTTTTTGCGTTTTTTCTTATAGCAAGGACCAGGCAGAAAGAAGGACAACTCATAAACCTTT
GTAATAGCAATCAGCGTCAGTACAACATATAATTCCACTTCAACAACGGATCTCTTGGTTCTGGCA
TCGATGAAGAACGCAGCGAATCGATACGTAGTTTGATGCAGAATTCAGTGAATCATCGAATCCTTG
AACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGC
TTTGCTGGTGTTGGGCGTCTTGTCTCCAGTCCGCTGGAGACTCGCCTTAAAGTCATTGGCAGCCGGC
CTACTGGTTTCGGAGCGCAGCACAAGTCGCACTCTTTTCCAGCCAAGGTCAGCGTCCAACAAGCCT
TTTTTCAACTTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGA
GGAAAAGAAACCAACAGGGATTGCCCTAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAA
ATCTGGCTCTTTTAGAGTCCGAGTTGTAATTTGCAGAGGGCGCTTTGGCTTTGGCAGCGGTCCAAGT
TCCTTGGAACAGGACGTCACAGAGGGTGAGAATCCCGTACGTGGTCGCTGGCTATTGCCGTGTAAA
GCCCCTTCGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAATGGGAGGTACATTTCTTCTAAAGC
TAAATATTGGCCAGAGACCGATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGGAA
AGAGAGTCAAACAGCACGTGAAATTGTTGAAAGGGAAGCGCTTGCAGCCAGACTTGCTTACAGTT
GCTCATCCGGGCTTTTGCCCGGTGCACTCTTCTGTAGGCAGGCCAGCATCAGTTTGGGCGGTAGGA
TAAAGGTCTCTGTCACGTACCTCTCCTTCGGGGAGGCCTTATAGGGGAGACGACATACTACCAGCC
TGGACTGCAGG3’

>Utah fungal isolate No. 20 (*Alternaria sp.* Utah 9)

5’ACAAGGTACTGCCGTAGGGTGAACCTGCGGGGGGGATCATTACACAAATATGAAGGCGGGCTGG
AACCTCTCGGGGTTACAGCCTTGCTGAATTATTCACCCTTGTCTTTTGCGTACTTCTTGTTTCCTTGG
TGGGTTCGCCCACCACTAGGACAAACATAAACCTTTTGTAATTGCAATCAGCGTCAGTAACAAATT
AATAATTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCG
ATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGG
TATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTCT
TGTCTCTAGCTTTGCTGGAGACTCGCCTTAAAGTAATTGGCAGCCGGCCTACTGGTTTCGGAGCGCA
GCACAAGTCGCACTCTCTATCAGCAAAGGTCTAGCATCCATTAAGCCTTTTTTTCAACTTTTGACCT
CGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAG
GGATTGCCCTAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCTCTTTTAGAGT
CCGAGTTGTAATTTGCAGAGGGCGCTTTGGCTTTGGCAGCGGTCCAAGTTCCTTGGAACAGGACGT
CACAGAGGGTGAGAATCCCGTACGTGGTCGCTGGCTATTGCCGTGTAAAGCCCCTTCGACGAGTCG
AGTTGTTTGGGAATGCAGCTCTAAATGGGAGGTACATTTCTTCTAAAGCTAAATATTGGCCAGAGA
CCGATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGGAAAGAGAGTCAAACAGCAC
GTGAAATTGTTGAAAGGGAAGCGCTTGCAGCCAGACTTGCTTACAGTTGCTCATCCGGGTTTCTAC
CCGGTGCACTCTTCTGTAGGCAGGCCAGCATCAGTTTGGGCGGTAGGATAAAGGTCTCTGTCACGT
ACCTCCTTTCGGGGAGGCCTTATAGGGGAGACGACATACTACCAGCCTGGACTGAGGTCCGCGCAT
CTGCTAGGGATGCTGGCGTAATGCTTAA3’

>Utah fungal isolate No. 21 (*Alternaria sp.* Utah 10)

5’CGAAACAAGGTACTCCGTAGGTGAACCTGCGGAGGGGATCATTACACAAATATGAAGGCGGGCT
GGAACCTCTCGGGGTTACAGCCTTGCTGAATTATTCACCCTTGTCTTTTGCGTACTTCTTGTTTCCTT
GGTGGGTTCGCCCACCACTAGGACAAACATAAACCTTTTGTAATTGCAATCAGCGTCAGTAACAAA
TTAATAATTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG
CGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTT
GGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGT
CTTGTCTCTAGCTTTGCTGGAGACTCGCCTTAAAGTAATTGGCAGCCGGGCCTACTGGTTTCGGAGC
GCAGCACAAGTCGCACTCTCTATCAGCAAAGGTCTAGCATCCATTAAGCCTTTTTTTCAACTTTTGA
CCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAA

CAGGGATTGCCCTAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCTCTTTTAG
AGTCCGAGTTGTAATTTGCAGAGGGCGCTTTGGCTTTGGCAGCGGTCCAAGTTCCTTGGAACAGGA
CGTCACAGAGGGTGAGAATCCCGTACGTGGTCGCTGGCTATTGCCGTGTAAAGCCCCTTCGACGAG
TCGAGTTGTTTGGGAATGCAGCTCTAAATGGGAGGTACATTTCTTCTAAAGCTAAATATTGGCCAG
AGACCGATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGGAAAGAGAGTCAAACA
GCACGTGAAATTGTTGAAAGGGAAGCGCTTGCAGCCAGACTTGCTTACAGTTGCTCATCCGGGTTT
CTACCCGGTGCACTCTTCTGTAGGCAGGCCAGCATCAGTTTGGGCGGTAGGATAAAGGTCTCTGTC
ACGTACCTCCTTTCGGGGAGGCCTTATAGGGGAGACGACATACTACCAGCCTGGACTGAGGTCCGC
GCATCTGCTAGGAGCTGGCGTAATGCTAACTTC3’

>Utah fungal isolate No. 22 (*Alternaria sp.* Utah 11)

5’CGGAACAAGGTACTCCGTAGGGTGAAACCTGCGGGGGGGATCATTACACAAATATGAAGGCGGG
CTGGAACCTCTCGGGGTTACAGCCTTGCTGAATTATTCACCCTTGTCTTTTGCGTACTTCTTGTTTCC
TTGGTGGGTTCGCCCACCACTAGGACAAACATAAACCTTTTGTAATTGCAATCAGCGTCAGTAACA
AATTAATAATTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA
TGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCT
TTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGC
GTCTTGTCTCTAGCTTTGCTGGAGACTCGCCTTAAAGTAATTGGCAGCCGGCCTACTGGTTTCGGAG
CGCAGCACAAGTCGCACTCTCTATCAGCAAAGGTCTAGCATCCATTAAGCCTTTTTTTCAACTTTTG
ACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCA
ACAGGGATTGCCCTAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCTCTTTTA
GAGTCCGAGTTGTAATTTGCAGAGGGCGCTTTGGCTTTGGCAGCGGTCCAAGTTCCTTGGAACAGG
ACGTCACAGAGGGTGAGAATCCCGTACGTGGTCGCTGGCTATTGCCGTGTAAAGCCCCTTCGACGA
GTCGAGTTGTTTGGGAATGCAGCTCTAAATGGGAGGTACATTTCTTCTAAAGCTAAATATTGGCCA
GAGACCGATAGCGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGGAAAGAGAGTCAAAC
AGCACGTGAAATTGTTGAAAGGGAAGCGCTTGCAGCCAGACTTGCTTACAGTTGCTCATCCGGGTT
TCTACCCGGTGCACTCTTCTGTAGGCAGGCCAGCATCAGTTTGGGCGGTAGGATAAAGGTCTCTGT
CACGTACCTCCTTTCGGGGAGGCCTTATAGGGGAGACGACATACTACCAGCCTGGACTGAGGTCCG
CGCATCTGCTAGGGATGCTGGCGTAATGCTAACT3’

>Utah fungal isolate No. 23 (*Alternaria sp.* Utah 12)

5’CGACAAGGAACCGCGCCGTGAACCTGCGGGGGATCATTACACAAATATGAAGGCGGGCTGGAAC
CTCTCGGGGTTACAGCCTTGCTGAATTATTCACCCTTGTCTTTTGCGTACTTCTTGTTTCCTTGGTGG
GTTCGCCCACCACTAGGACAAACATAAACCTTTTGTAATTGCAATCAGCGTCAGTAACAAATTAAT
AATTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATA
AGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATT
CCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTCTTGTC
TCTAGCTTTGCTGGAGACTCGCCTTAAAGTAATTGGCAGCCGGCCTACTGGTTTCGGAGCGCAGCA
CAAGTCGCACTCTCTATCAGCAAAGGTCTAGCATCCATTAAGCCTTTTTTTCAACTTTTGACCTCGG
ATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGA
TTGCCCTAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCTCTTTTAGAGTCCG
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