Nonrandom mating in *Nicotiana attenuata* and the paternal influence on seed metabolomes and pathogen resistance

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Abbreviations

AMF	Arbuscular mycorrhizal fungi
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
ESI/HR-Q-TOF	Electrospray ionization/ high resolution quadrupole time-of-flight
ESI/TOF	Electrospray ionization/ time-of-flight
EV	Empty vector
GA_3	Gibberellic acid
GB5	Gamborg's B5 medium
GLM	Generalized linear model
GUS	β -Glucuronidase
ITS	Internal transcribed spacer
LSM	Laser scanning microscope
LSU	Large ribosomal subunit
MS	Mass spectrometry
PC	Principal component
PCA	Principal component analysis
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PPEB	Post-pollination ethylene burst
PrpS	*Papaver rhoeas* pollen S
PrsS	*Papaver rhoeas* style S
PT	Pollen tube
RNA	Ribonucleic acid
RNase	Ribonuclease
UHPLC	Ultrahigh-performance liquid chromatography

1. Introduction

1.1 Sexual selection and its application in plants

In *The Descent of Man* (Darwin, 1871), Darwin adopted Hunter's distinction between primary and secondary sexual characters (Hunter, 1780, 1837) and first coherently explained elaborate weapons and ornaments found in many male animals and less commonly in females. In his book, secondary sexual characters were defined by Darwin as characters used in intrasexual competition to breed. Darwin termed the evolutionary process generating those characters "sexual selection". In his book (Darwin, 1871), he suggested two mechanisms of sexual selection: "In the one it is between individuals of the same sex, generally the males, in order to drive away or kill their rivals, the females remaining passive; whilst in the other, the struggle is likewise between individuals of the same sex, in order to excite or charm those of the opposite sex, generally the females, which no longer remain passive, but select the more agreeable partners." Darwin's theory of sexual selection was close to the modern version of intrasexual competition, and he correctly realized the importance of intersexual choice which is a major aspect of sexual selection (Jones and Ratterman, 2009). Overall, Darwin's theory of sexual selection is essentially similar to the modern definition of pre-copulatory sexual selection in animals.

In 1970, Parker proposed the concept of sperm competition, which is nowadays considered as the post-copulatory equivalent of intrasexual competition. He, in his paper, also explained the evolutionary logic of sperm competition and set the agenda for future studies (Parker, 1970). Later, Thornhill (1983, 1984) referred to the post-copulatory equivalent of female choice as cryptic female choice, which described any post-copulatory ability of females to favor one conspecific male over another. The definition of sexual selection had been broadened from pre-copulatory to post-copulatory stages in animals. However, Darwin himself barely mentioned plants in his book about sexual selection. He, in fact, did not even consider lower animal phyla, since he believed that the primitive organisms could not engage in sexual selection. Furthermore, most plants are hermaphrodites and lack secondary sexual characters. All these,

perhaps, hindered the application of sexual selection theory to the kingdom of plants, until late 1970s and early 1980s (Willson, 1979; Queller, 1983).

Early efforts from botanists to apply sexual selection theory to plants were met with skepticism. In 1994, there was a symposium issue focused specifically on the question of whether a unifying concept of sexual selection in plants and animals exists (Arnold, 1994a, b). After that, Grant (1995) suggested that the division of opinions and the most critical arguments for and against sexual selection in plants were mainly based on the definition of sexual selection. As Arnold (1994b) proposed, a general definition of sexual selection was desired, a definition that does not rule out the possible existence of sexual selection in other groups of organisms, such as plants. Arnold (1994b) did give a generalized definition, as he stated: “Sexual selection is selection that arises from differences in mating success (number of mates that bear or sire progeny over some standardized time interval).” A similar definition was offered by Stanton (1994). Grant (1995) commented: “The definitions of Arnold and Stanton are on the right track, insofar as they put the focus on mating success, but are not quite restrictive enough.” However, as more phenomena are being unearthed, the definition of sexual selection, today, is even less restrictive, and more along the lines of the broader definition proposed by Willson (1994), which expanded Darwin’s original concept: “to encompass all events related to sexual competition and mate choice whenever they occur in the reproductive sequence, from pre-courtship to postnatal phases. For example, sperm competition and gametophyte competition become part of sexual selection, as I use the term, because conceptually they are forms of intermale competition.” As the definition of sexual selection has been extended, today the application of this theory in plants is accepted among botanists as a phenomenon which can occur before pollination, after pollination but before fertilization and after fertilization (Skogsmyr and Lankinen, 2002; Moore and Pannell, 2011; Lankinen and Green, 2015).

1.2 Sexual selection in plants

In flowering plants, pre-pollination sexual selection refers to the influence of traits affecting pollinator visitation, or the efficiency of pollen transfer by pollinators or other bearers,

on the transfer of pollen to conspecific stigmas. In *Polemonium viscosum*, the wider the corolla flare, the more pollen was removed by bumble bees, and the more exserted the style, the more pollen was received by the stigma (Galen and Stanton, 1989). In *Cypripedium acaule*, one individual plant normally produces one flower every year. The flower does not offer any reward to the visitors. Since the pollinators may learn to avoid the "cheating" flowers in the late flowering season, earlier dates of flower opening were selected for (O'Connell and Johnston, 1998). Cocucci *et al.* (2014) proposed intrasexual competition mediated through physical male interference in milkweeds (Apocynaceae, subfamily Asclepiadoideae): when more than two pollinaria picked by one pollinator stuck together to form a so called pollinarium chain, the distal pollinarium might deliver the pollinia without interference. On the contrary, the proximal pollinarium with respect to the pollinator could deliver the pollinium with interference from the other pollinarium attached on it.

Post-pollination sexual selection occurs when pollen from more than one donor lands on the stigma, and seeds are sired disproportionately to the amounts initially present on the stigma. Post-pollination sexual selection consists of two aspects of selection: pre-zygotic and post-zygotic selection. Post-zygotic selection results in the abortion of fruits and seeds before they reach maturity. Many factors affect the abortion of ovules and flowers, such as availability of nutrients, the position of the flowers and ovules, the order of pollination on the flowers and genetic factors of the embryos (Westoby and Rice, 1982; Lee and Bazzaz, 1986; Charlesworth, 1989; Rocha and Stephenson, 1991; Kärkkäinen *et al.*, 1999; Susko and Lovett-Doust, 1999). In many cases, the abortion of the embryos is nonrandom. In *Erythronium grandiflorum*, ovules fertilized by self pollen or pollen donors closer to the maternal plants were aborted more than ovules fertilized by distant pollen donors (Rigney, 1995). Selective embryo abortion was an important approach to reduce the proportion of low-quality offspring in *Cynoglossum officinale*, and thus the offspring remaining after more severe abortion had higher survival (Melser and Klinkhamer, 2001).

Introduction

The life cycle of flowering plants provides a rich potential for pre-zygotic sexual selection. It is the stage when pollen tubes (PTs) and pistils are interacting with each other. When pollen reaches the stigma of the pistil, PTs germinate from compatible and hydrated pollen grains. The subsequent growth of each PT generates a tip-growing cell burrowing through the style and delivering the sperm cells into the embryo sac in the ovule protected by the ovary. The stage before sperms fuse with the egg and the two polar nuclei in the central cell is defined as the pre-zygotic stage. In this stage, due to the lack of nutrients and other resources to support its own growth, each PT must be sustained and nursed by the pistil before it reaches the destiny of the long journey, the egg and the central cell in the embryo sac. Therefore, the pistil, standing between pollen receipt and the double fertilization, may function as a gatekeeper, facilitating the growth of favored PTs and hindering incompatible and less desirable pollen (Heslop-Harrison, 2000; Higashiyama, 2010; Bedinger *et al.*, 2017).

Self-incompatibility systems, one mode of pre-zygotic mate choice, have drawn much attention. Many flowering plant species use self-incompatibility mechanisms to prevent self-fertilization. These consist of recognition of self versus non-self pollen and subsequent selective inhibition on the self PT development. Three types of self-incompatibility have so far been discovered: Brassicaceae type, Solanaceae type and Papaveraceae type. Brassicaceae-type self-incompatibility is sporophytic self-incompatibility, where the pollen self-incompatible phenotype is determined by the diploid sporophyte (anther) in which the pollen develops. In plants with this type of self-incompatibility, pollen grains fail to develop into PTs when the male determinant (a small protein) in the pollen coat can be recognized by the female determinant (a receptor kinase) expressed in stigma papilla cells. Solanaceae-type and Papaveraceae-type self-incompatibilities are gametophytic self-incompatibilities, where the self-incompatible phenotype is determined by the haploid genotype of pollen, and thus the rejection of self pollen happens during PT growth. In plants with Solanaceae-type self-incompatibility, ribonucleases expressed in the style (S-RNases), the female determinant, penetrate into the growing PTs and function as cytotoxins that degrade RNA in PTs. In non-self PTs, the male determinant (F-box family of proteins) triggers ubiquitin-mediated protein degradation of non-

self S-RNases. Hence, only non-self PTs can grow through the style and complete fertilization. In Papaveraceae-type self-incompatibility, programmed cell death (PCD) in pollen results in self-rejection. The female determinant is a small protein called PrsS (*Papaver rhoeas* style S) secreted by stigmatic papilla cells. The male determinant is also a small protein called PrpS (*P. rhoeas* pollen S) on the PT membrane. The PrsS protein interacts with the PrpS protein from the self PT and induces Ca^{2+} influx in PT. The increase of Ca^{2+} results in PCD of the self PT (Takayama and Isogai, 2005; Wilkins *et al.*, 2014; Fujii *et al.*, 2016).

Furthermore, pre-zygotic nonrandom mating can also happen among pollen from compatible donors (Currah, 1981; Marshall and Ellstrand, 1986; Skogsmyr and Lankinen, 1999). In the dioecious plant, *Silene alba*, males producing offspring with biased sex ratios sired far fewer offspring of either sex when in pollen mixtures collected from different males producing offspring with various sex ratios. However, all these males produced similar amount of offspring in single pollinations (Taylor *et al.*, 1999). Carlson *et al.* (2009) discovered that nonrandom mating among distinct compatible accessions occurs in *Arabidopsis thaliana*. Abundant studies on various plant species suggested that nonrandom mating often occurs among compatible donors, yet the mechanisms are still poorly understood.

1.3 Nonrandom mating among compatible donors in *Nicotiana attenuata*

Nicotiana attenuata Torr. Ex Watts. (Solanaceae) is an annual self-compatible diploid native tobacco in the South-western USA (Sime and Baldwin, 2003). It germinates after fires from long-lived seed banks (Preston *et al.*, 2002), and thrives in genetically diverse populations (Bahulikar *et al.*, 2004). *N. attenuata* is a self-compatible opportunistic outcrosser (Sime and Baldwin, 2003; Kessler *et al.*, 2008). Its white flowers consist of petals fused into tubular corollas with zygomorphically symmetric limbs. The flowers primarily open in the evening and remain open for 3 days. Nocturnal hawk moths, *Manduca quinquemaculata* and *Manduca sexta*, and diurnal hummingbirds are the most often observed pollinators of *N. attenuata* (Kessler *et al.*, 2008).

Bhattacharya and Baldwin (2012) discovered the presence of nonrandom mating by maternal plants (UtWT, collected from Utah, USA, inbred in the greenhouse) when mixed pollinations with two pollen donors were conducted. Bhattacharya and Baldwin mixed equal amounts of self pollen (UtWT) and one of the cross pollen donors (G2 or G10, collected from Utah, USA), and then performed mixed pollination on UtWT plants. Genotyping of offspring seedlings indicated that UtWT pollen consistently sired more seeds in the seed set compared with the non-self pollen donors. Aside from this, although cross pollen sired less seeds than self pollen, the G2 pollen donor sired a greater proportion of seeds in the seed set than the G10 pollen donor did. Therefore, the G2 pollen donor was defined as a favored non-self pollen donor while the G10 pollen donor was considered as a non-favored non-self pollen donor. However, in single pollinations all pollen of different genotypes tested had equal fecundity and sired an equal number of seeds per capsule. Besides, they also demonstrated that ethylene signaling was involved in this pre-zygotic nonrandom mating process. The post-pollination ethylene burst (PPEB) from UtWT flowers pollinated with the favored non-self G2 pollen was higher than the PPEB from UtWT flowers pollinated with the non-favored non-self G10 pollen. The flowers from two transgenic genotypes with ethylene signaling deficiencies lost the ability of nonrandom mating (irACO, silenced in 1-aminocyclopropane-1-carboxylic acid oxidase activity, cannot produce ethylene and ETR1, overexpressing a mutated ethylene receptor, cannot perceive ethylene, both lines are transformed from UtWT). Since Bhattacharya and Baldwin did not find any evidence suggesting nonrandom seed abortion, they believed the nonrandom mating in *N. attenuata* is pre-zygotic.

Furthermore, Bhattacharya and Baldwin (2012) tried to identify the area where the nonrandom mating occurs in the style. Since UtWT recognized an empty vector (EV, the empty vector transformant from UtWT with hygromycin B resistance as the transformation selectable marker) as a non-self pollen donor, Bhattacharya and Baldwin developed a semi-*in vivo* system with UtWT+EV mixed pollen combination on detached UtWT styles cut at different distances away from the stigmas. Since the EV PTs can grow on the medium with hygromycin B while UtWT PT cannot, by comparing the number of PTs emerging from stylar cut ends, they revealed

that the discrimination of the numbers of PTs between UtWT and EV started at 6 mm from the stigma and became more prominent as the distance increasing (the relevant previous findings from Bhattacharya and Baldwin are reviewed in Figure 1). However, since the semi-*in vivo*

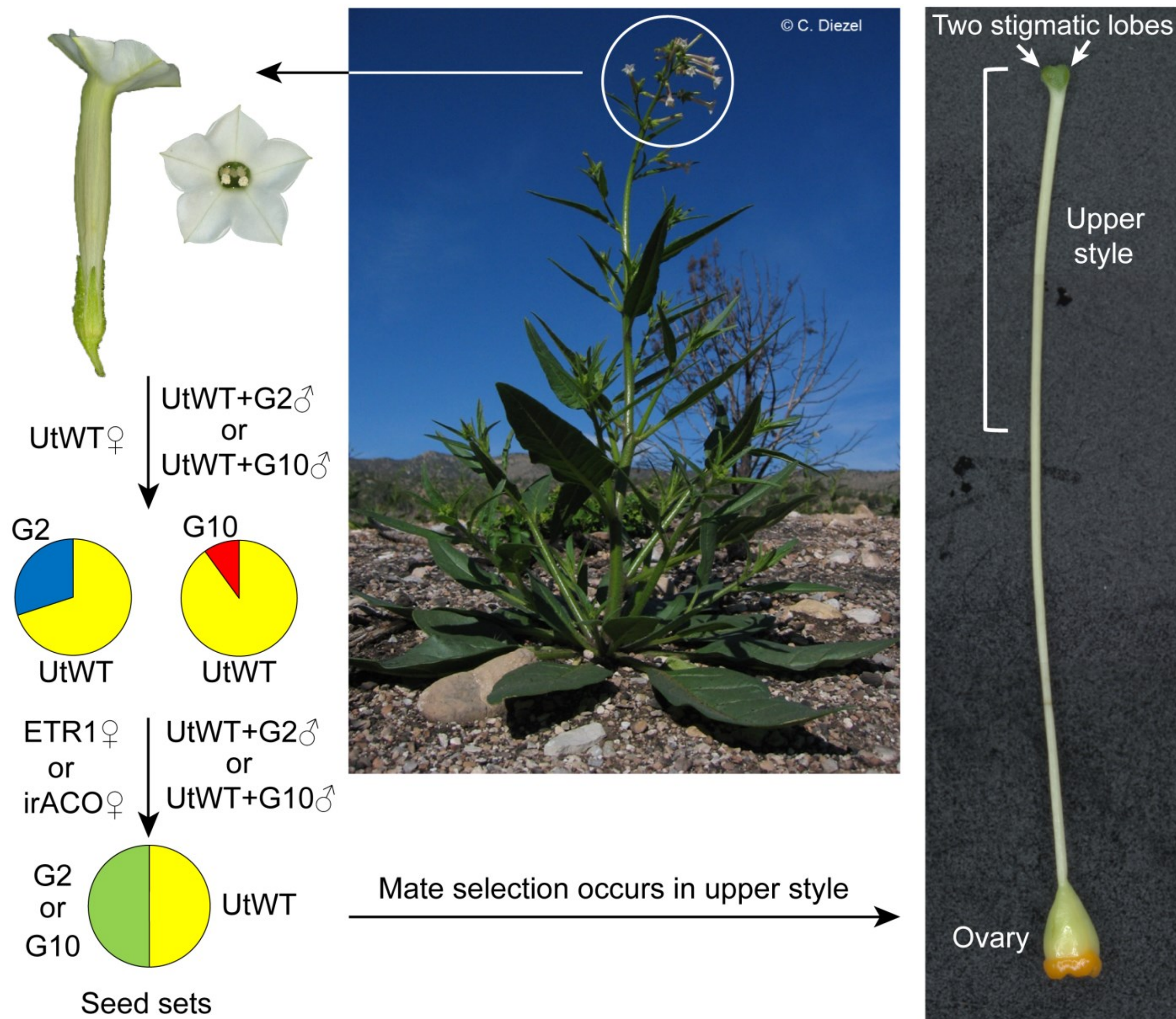


Figure 1. Nonrandom mating in *Nicotiana attenuata*. UtWT maternal plans selected for self pollen, when mixed pollination was conducted. G2 (favored non-self pollen donor) pollen fertilized higher proportion of eggs in a capsule than G10 (non-favored non-self pollen donor) pollen did, when binary mixed pollinations with self pollen were performed. Ethylene-deficient lines, irACO (transformed line that cannot produce ethylene) and ETR1 (transformed line that cannot perceive ethylene), lost the ability of pre-zygotic mate selection. Therefore, the pollen donors used in mixed pollination produced averagely equal amounts of seeds in the seed set. It was demonstrated that mate selection in *N. attenuata* occurred in upper style. The picture with the whole plant of *N. attenuata* was taken in Utah by Diezel.

system is still an artificial system and only the numbers of the PTs emerging at the ends of styles were recorded, the linkage between the *in vivo* PT growth rates of different pollen donors and their paternal fecundities in mixed pollination is still elusive. The difference in the numbers of the PTs emerging at the ends of styles may be due to differential germination of pollen grains rather than the differential growth rates of the PTs. Because the semi-*in vivo* system used in a previous study also suggested that the genetically transformed pollen may affect seed set after nonrandom mating, a new method to track PT growth *in vivo* by avoiding using transformed pollen need to be developed for further understanding of the PT growth in mixed pollination and if it is involved in nonrandom mating in *N. attenuata*.

1.4 PT growth rates and stylar metabolites in nonrandom mating

Jones (1922) first proposed that differential PT growth rates are a potential cause of the phenomenon that pollen from different donors sires seeds disproportionate to the amounts initially presenting on the stigma. Theoretically, the unfertilized ovules are more likely to be fertilized by faster-growing PTs, and this idea has been supported by quite a few studies. In some instances, faster *in vitro* PT growth rates reflect the success in mixed pollinations (Aronen *et al.*, 2002; Lankinen and Skogsmyr, 2002; Figueroa-Castro and Holtsford, 2009). However, compared with *in vivo* PT growth, PTs growing *in vitro* do not achieve the high speed and the length of growth (Read *et al.*, 1993a, b). Therefore, *in vitro* PT growth rates can partially indicate the rate of *in vivo* growth where the biochemical, physiological and physical environment of the pistil is present.

In order to obtain PT performance closer to the real situation in mixed pollination, other studies chose to record *in vivo* PT growth rates in single pollinations, and thus to predict *in vivo* PT growth rates in mixed pollinations, and further to positively correlate with the seed-siring (Pasonen *et al.*, 1999; Marshall and Diggle, 2001; Pasonen *et al.*, 2002). However, this method is unable to inspect the interactions between PTs from different donors in mixed pollination. Furthermore, this method is limited to reveal the effects of maternal choice on different PTs from the competitive pollen donors in mixed pollination. Therefore, some studies tried to view

the differential PT growth in mixed pollination by using the morphology of the pistils. For example, in species with separate styles or stigmas that converge to the same ovary, such as *Dianthus chinensis* or *Hibiscus moscheutos*, PT growth rates of different donors were measured within the same flower pollinated by loading pollen from competitive donors separately on the separate stigmas (Aizen *et al.*, 1990; Snow and Spira, 1991). As for species with different channels in a hollow style, such as *Erythronium grandiflorum*, pollination was done by placing different pollen separately on different lobes of the same stigma, and after that, PT growth rates of competitive donors within the same pistil were measured (Cruzan, 1990). In some studies, genetically transformed pollen and PTs were used to track the differential growth of different pollen donors in mixed pollination. Swanson *et al.* (2016) transformed one genotype of pollen with a colorimetric marker β -glucuronidase (GUS) reporter gene in the binary mixed pollination, while the other genotype of pollen in the pollination was left unlabeled. By applying this method, the *in vivo* differential PT growth rates in the mixed pollination were measured to demonstrate that pollen donor with faster growing PTs has a greater chance to sire the seeds. However, all these methods are not suitable in *N. attenuata*. As mentioned above, genetically transformed *N. attenuata* pollen may affect the nonrandom mating process (Bhattacharya and Baldwin, 2012). Therefore, in this research, genetically transformed pollen was avoided to achieve unbiased results. A pistil of *N. attenuata* has two fused carpels (a unified compound gynoecium, syncarpy) with one solid style and one stigma with two lobes. Hence, it is challenging to view differential PT growth rates of different pollen donors within the same style in mixed pollination. Nevertheless, in this research, I developed a method for the first time to view the *in vivo* differential PT growths of different pollen donors within the same style of a unified compound gynoecium.

Aside from the *in vivo* tracking of PT growth rates of different pollen donors within the same style, the secondary metabolites related to pre-zygotic mate selection in *N. attenuata* remain unknown. The understanding of the metabolomics changes of pistils related to pollination and maternal selection may help to reveal the mechanism of the pre-zygotic mate selection in *N. attenuata*. Most extant studies focused on the stylar peptides, proteins and

lipids involved in PT-pistil interaction or sexual selection (Hiscock and Allen, 2008; Higashiyama, 2010; Chae and Lord, 2011; Pereira *et al.*, 2016). Few studies paid attention on the stylar secondary metabolites (Zhao *et al.*, 2016). Zhao *et al.* (2016) generally reported the difference of metabolomes between self-compatible and self-incompatible tomato styles. However, they failed to conclude which metabolites are involved in the nonrandom mating process. Therefore, I conducted metabolomics analyses on the styles after different mixed pollinations. I tried to answer the question of which secondary metabolites are involved in mate selection in *N. attenuata*.

1.5 Paternal effects on offspring qualities and seed metabolites

PT is heterotrophic, and the pistil where it grows supplies the nutrients for it and interacts with it. During the journey to the ovules, many genes are expressed in PTs. As reported in *Arabidopsis*, 7044 genes were expressed in PTs growing through stigma and style in a semi-*in vivo* system, while 12624 genes were expressed in the sporophyte (seedlings, roots, leaves and unpollinated stigmas and ovaries) (Qin *et al.*, 2009). Among the genes expressed in PTs, many of them were also detectable in seedlings (Ottaviano and Mulcahy, 1989; Qin *et al.*, 2009). Therefore, it is reasonable to expect that the successful paternal genotype in pre-zygotic mate selection may produce offspring with higher quality, compared with the unsuccessful paternal genotype. In *Raphanus sativus*, the most successful pollen donor in nonrandom mating sired offspring with largest growth, which suggested that sorting among paternal lines may have improved the quality of the offspring (Marshall, 1988; Marshall and Whittaker, 1989). As mentioned previously, sexual selection may also occur after fertilization. Post-zygotic mate choice may alter the quality of offspring, as well. *Lotus corniculatus* selectively aborted fruits with the fewest seeds, which were possibly sired by pollen with low quality, and thus the average quality of the offspring was increased with more vigorous seedlings and more reproductive adults (Stephenson and Winsor, 1986). In fact, nonrandom mating in plants need not directly improve offspring growth or quality (Arnold, 1983). Rather, successful pollen donors in sexual selection might simply affect the mating characteristics of their offspring, such as number of pollen grains or PT growth rate. Even female choice need not improve offspring

growth or quality, if the choice is based on characters related to pollen performance (Marshall and Whittaker, 1989). However, many scientists were fascinated with the question of whether different paternities influence the quality of their offspring. Evidence from many studies suggested that paternity might influence on the progeny fitness (Antonovics and Schmitt, 1986; Waser and Price, 1989; Andersson, 1990; Pasonen *et al.*, 2001).

Seeds are offspring that firstly leave the maternal plants. Seeds are fundamentally crucial to plants, because they constitute the main method of plant propagation. For annual plants, seeds are even more crucial. The qualities of these seeds influence if their genomes obtained from their parents will be passed to the further generations. Some studies suggest that there are paternal effects on seed traits and qualities, while others do not. Andersson (1990) showed that, in annual *Crepis tectorum*, pollen donors influenced seed weight and seed width. In *Raphanus sativus*, paternity affected mean seed weight (Nakamura and Stanton, 1989). On the contrary, Pelabon *et al.* (2015) discovered that, in *Dalechampia scandens*, paternal identity did not affect seed mass. In molecular level, it was demonstrated that, in *Arabidopsis thaliana*, there was a large pool of cryptic genetic variation in the paternal control of seed development, whose effects were buffered by maternal regulators of seed development (Pires *et al.*, 2016). This might be one of the reasons why paternal effects on seed traits are considered equivocal. Most of the extant studies related to the paternal effects on seeds focus on the seed weight, seed size or seed development. However, few studies try to address the paternal effects on seed metabolite profiles. Seed metabolites are important for longevity of seeds, seed germination, seed defense, etc. As a factor of seed quality, seed metabolites are very likely to be impacted by paternal identity.

N. attenuata is an annual desert plant whose seeds lay dormant in seed banks, and it was inferred that *N. attenuata* seeds could remain dormant in a long-lived seed bank for about 150 years (Preston and Baldwin, 1999). Preston and Baldwin (1999) found an area of the natural habitat dominant with juniper trees, and some of them were around 150 years old. Fire is fatal to junipers and they do not re-sprout after being burned (Wright *et al.*, 1979). Therefore,

Preston and Baldwin (1999) deduced there was no fire in the area for about 150 years. They discovered no *N. attenuata* plants in the area for 8 consecutive years, and no population of *N. attenuata* close by, and no known seed dispersers of it. Nevertheless, after fire killed all the fire-sensitive junipers, a large population of *N. attenuata* was found growing in the area. Since *N. attenuata* germinates after fire, it was inferred that there may be a long-lived seed bank. It remains unknown that if the paternity affects seed metabolite profiles in *N. attenuata*. If so, a new question will be opened: Do different metabolite profiles of seeds confer the seeds with different performance in the seed bank? Investigating the paternal effects on seed metabolomes will provide knowledge not only about the paternity importance, but also about the potential diversity of the seeds in native seed banks. In this study, 26 different hybrid seeds, obtained from hand pollination on two different maternal lines (11 paternal lines on one maternal line, and 15 paternal lines on the other maternal line) in greenhouse, were used to investigate whether different paternal identities can impart their seeds with different metabolite profiles.

1.6 The interaction between seeds and soil microbes and roles of seed metabolites

Seeds, once entering into the seed banks, interact with various soil-borne microbes. Soil is a highly complex system which consists of numerous discrete microhabitats and unevenly distributed microbes (Nunan *et al.*, 2002; Mummey and Stahl, 2004). Physically disturbing soil, such as via insect movement, actions of burrowing animals, drying-wetting or freezing-thawing cycles, would presumably cause redistribution of seeds (Chambers and MacMahon, 1994; Westerman *et al.*, 2006) and thus may affect the chance of encounters between seeds and microbes. Many of these microbial taxa have no negative effects on seeds (Schafer and Kotanen, 2004). Seeds exude water-soluble carbohydrates and amino acids, which can stimulate fungal spore germination (Harman, 1983) or function as chemo-attractants for microbes (Caetano-Anollés *et al.*, 1988; Shingler, 2003). These microbes feed on many substrates around them and sometimes feed on seeds, by excreting lytic enzymes involving in substrate breakdown and degradation (Céspedes *et al.*, 1997; Lyons *et al.*, 2003; Martínez *et al.*, 2005). In addition, some microbes can kill or damage seeds via the release of phytotoxins prior

to seed germination (Harman, 1983; Kremer *et al.*, 1990). On the contrary, some microbes are considered as beneficial. The presence of nonpathogenic beneficial bacteria has been discovered to be able to shelter seeds from fungal attack (Anderson *et al.*, 1980; Hadar *et al.*, 1983; Kremer, 1987).

Among the culturable and well described soil-borne microbial taxa, *Fusarium*, a common fungal genus, causes plant diseases and seed decay in many plant species. *Fusarium* could cause seed deterioration in *Abutilon theophrasti* (Kremer and Schulte, 1989). *Fusarium* also was demonstrated to cause seed death of *Bromus*, *Danthonia* and *Poa* (Schafer and Kotanen, 2004). *Alternaria* is another well-known saprophytic and pathogenic fungal genus often found in soil. Over 100 species of *Alternaria* have been described (Simmons, 1992). The fungi from this genus can survive as mycelia or spores on dead plant tissues for a considerable time, or as a latent infection in seeds (Rotem, 1994). Cutinases, lipases and fungal galacturonidases can be secreted by many species of *Alternaria* to facilitate direct fungal penetration into the host tissue by degrading cuticles and other cell wall components (Trail and Köller, 1993; Yao and Köller, 1994; Yao and Köller, 1995; Berto *et al.*, 1997; Isshiki *et al.*, 2001). *Alternaria* species are also known as seed pathogens in many plant species, and thus have various impacts on seed bank persistence and dynamics (Kirkpatrick and Bazzaz, 1979; Pitty *et al.*, 1987; Schafer and Kotanen, 2004).

Aside from deleterious soil-borne microbes, there are beneficial microbes as well. Many terrestrial orchids have associations with fungi and their seeds often require facilitation by suitable fungi of germination and seedling establishment (Zelmer *et al.*, 1996; Zettler and Hofer, 1998). Legumes are known to establish symbiotic associations with a group of bacteria, *Rhizobium*, which fix atmospheric nitrogen in exchange for photosynthates (Deaker *et al.*, 2004). Among the known soil-borne beneficial microbes, fungi in the genus *Trichoderma* have been known as biocontrol agents for the control of plant diseases since the 1930s (Weindling, 1932). The principal biocontrol mechanisms employed by *Trichoderma* are via mycoparasitism (illustrated in Figure 2) (Papavizas, 1985; Howell, 2003) antibiosis (Howell, 1998;

Sivasithamparam and Ghisalberti, 1998) and inducing plant systemic disease resistance (Howell, 2006; Shoresh *et al.*, 2010). Mycoparasitism is a phenomenon where a parasitic fungus parasitizes a host fungus via forming appressorium-like structures and hook-shaped contact branches or coiling around the host fungal hyphae (Dennis and Webster, 1971; Chet *et al.*, 1981). In the past 20 years, *Trichoderma* spp. have been increasingly and successfully applied world-wide to control plant diseases in agriculture (Harman, 2000; Harman *et al.*, 2010). Aside from the biocontrol effects on seedlings and established plants, some species of *Trichoderma* also provide significant biocontrol effects against seed rot (Hadar *et al.*, 1984).

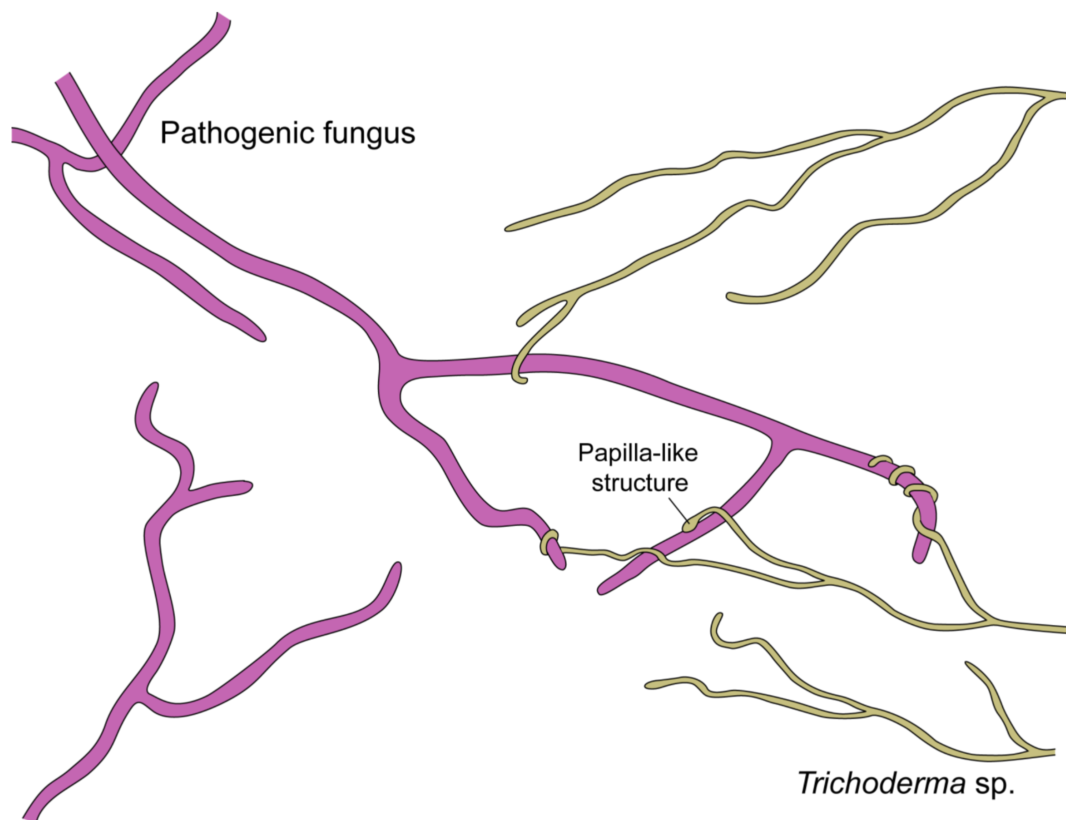


Figure 2. Depiction of mycoparasitism of *Trichoderma* spp. on pathogenic fungus. In soil community, *Trichoderma* spp. recognize a host fungus, and then coil around the hyphae of the host. Via mycoparasitism, *Trichoderma* spp. get nutrients from the host and eventually cause the death of the host fungus.

Seeds produced a large variety of metabolites, and during the interaction between seeds and microbes, metabolites of seeds often play various important roles. Some of the metabolites

function as attractants for beneficial microbes, while others are involved in seed defense mechanisms against deleterious microbes. In legumes, a flavone, luteolin, released by hydrated seeds can function as chemo-attractants for rhizobia (Caetano-Anollés *et al.*, 1988; Ndakidemi and Dakora, 2003). Although the chemical signaling between orchid seeds and suitable mycorrhizal fungi is still elusive, it is speculated that metabolites from orchid seeds may play roles in active attraction and recognition of compatible fungi (Weston *et al.*, 2005). Gowland *et al.* (2007) demonstrated that mycorrhizal fungi are attracted by viable orchid seeds. However, the chemistry involved in this chemotropism is still unclear. Similar to the interaction between orchid seed and its mycorrhizal fungi, the chemical communication between plant and *Trichoderma* is still poorly understood. Root exudates are considered as the main attractants for *Trichoderma* colonization (Druzhinina *et al.*, 2011). However, the question whether seed metabolites are implicated in the recruitment of *Trichoderma* remains unanswered.

Since seeds can be dormant structures, many defense compounds in mature seeds are constitutive. In *Cercidium floridum*, a species of palo verde, seed coat metabolites are involved in seed defense against seed predators and fungi (Siemens *et al.*, 1992). Imbibed cowpea seeds (*Vigna unguiculata*) exude defense proteins that inhibit the growth of a *Fusarium* fungal pathogen *in vitro* (Rose *et al.*, 2006). Cell wall located anti-fungal proteins in *Raphanus sativus* seeds can be released to the environment during germination, and thus provide a safe microenvironment for the germination seeds (Terras *et al.*, 1995). Other metabolites from seeds, such as soluble sugars, flavonoids and sterols have been discovered with defense activities (Casey *et al.*, 1998).

In *N. attenuata*, native fungal pathogens, such as species of *Alternaria* and *Fusarium*, cause diseases (Schuck *et al.*, 2014). Among them, sudden wilt disease is a root disease causing severe mortality in the field. Santhanam *et al.* (2015) reveal that a consortium of five root-associated bacteria is essential for providing the plants with resistance against this fungal disease. Furthermore, roots of *N. attenuata* can recruit arbuscular mycorrhizal fungi (AMF) and establish symbiosis (Riedel *et al.*, 2008; Groten *et al.*, 2015). However, it is unclear that how seeds

interact with the soil-borne fungi. It is also unknown that what seed metabolites are potentially related to the resistance against fungal attack. If different fathers can confer seeds with different seed metabolomes, it would be interesting to test whether seeds with different paternities have different pathogen resistances and which seed metabolites are involved in the differential pathogen resistance. In this study, native fungal pathogens isolated from Arizona were used to infect *N. attenuata* hybrid seeds with different paternal identities *in vitro*, in order to test whether different hybrid seeds have different fungal resistances. Analyses on metabolites of selected hybrid seeds were performed to unveil the metabolites potentially related to fungal pathogen resistance. Further, a native *Trichoderma* species was used to test its biocontrol ability on the seed pathogens. The findings will be essential for future studies to understand whether and how *N. attenuata* seeds interact with beneficial *Trichoderma*.

2. Aims of the thesis

Firstly, as introduced in Section 1.3, the previous study on *N. attenuata* has shown that it has nonrandom mating (Bhattacharya and Baldwin, 2012). However, it is unknown that if the *in vivo* differential PT growth rates of different pollen donors are involved in nonrandom mating. Although ethylene signaling was discovered to be involved in the nonrandom mating process (Bhattacharya and Baldwin, 2012), it is unclear that if any secondary metabolites is implicated in the process. Therefore, this study tried to develop a method to distinguish PTs from two pollen donors *in vivo* without genetic transformation on pollen, in order to demonstrate if *in vivo* differential PT growth rates of different pollen donors in mixed pollination are involved in nonrandom mating. Furthermore, in this study, I tried to find the secondary metabolites that are potentially involved in the nonrandom mating process.

Secondly, it remains unknown that if the paternal identity affects seed metabolomic profiles in *N. attenuata*. Investigating the paternal effects on seed metabolomes will provide knowledge not only about the paternity importance, but also about the potential diversity of the seeds in the native seed bank. In this study, 26 different hybrid seeds, obtained from hand pollination on two different maternal lines (11 paternal lines on one maternal line, while 15 paternal lines on the other maternal line) in greenhouse, were used to investigate whether different paternal identities can impart their seeds with different metabolomic profiles.

At last, in this study, native fungi were isolated from Arizona and Utah for further pathogenicity tests. The selected pathogens from Arizona were used to infect *N. attenuata* Arizona hybrid seeds with different paternities *in vitro*, in order to test if different hybrid seeds have different fungal resistances. Non-targeted analyses on metabolites of selected hybrid seeds were performed to unveil the metabolites potentially involved to differential fungal pathogen resistances. Further, a *Trichoderma* species obtained from Arizona was used to test its biocontrol ability on the selected fungal pathogens. The findings are useful for further research on seed-*Trichoderma*-pathogen interactions.

3. Materials and methods

3.1 *In vivo* pollen tube competition and secondary metabolites implicated in nonrandom mating of *Nicotiana attenuata*

3.1.1 Plant material and growth conditions

Nicotiana attenuata Torr. Ex Watts. seeds were originally collected from a plant growing in a large natural population (> 100,000 plants) near Santa Clara, Utah, USA, and inbred for 31 generations in the greenhouse (UtWT) (Glawe *et al.*, 2003). The 31st generation of UtWT plants was used in all hand-pollination experiments. Two additional native genotypes collected in Utah (G2 and G10; described in Bhattacharya and Baldwin, 2012) were selected as additional pollen donors. Ethylene biosynthesis-deficient transgenic line irACO and ethylene-insensitive line ETR1, derived from UtWT, were used as additional maternal plants. The genetically transformed lines have been fully characterized in von Dahl *et al.* (2007).

Seeds were germinated as described by (Krügel *et al.*, 2002). Plants were grown in one-liter pots at day temperature 23 – 25 ˚C under 16 h of light supplementation by Sun-T Agro 400 and 600 W sodium lights (Philips, Amsterdam, The Netherlands), and night temperature 19 – 23 ˚C. Plants were grown under uniform conditions and more details of the growing conditions such as soil type, fertilizing, potting and watering, were described in Bhattacharya and Baldwin (2012).

3.1.2 Emasculation and pollination

Flowers of 45 – 60 days old plants (UtWT, irACO and ETR1) were emasculated by antherectomization in the morning in the way described in Kessler *et al.* (2008) before they opened in the evening on the same day. The corollas of flowers were cut open and the anthers were gently removed. All the plants for pollination were used for no longer than 20 days after the first flower appeared. The emasculated flowers were pollinated or left unpollinated. For single pollination, several anthers from different flowers of the same genotype of plants were rolled onto a stigma. For mixed pollination, since Bhattacharya and Baldwin (2012) determined that the mean number of pollen grains per anther and their viability did not differ among the

genotypes used here, homogeneous pollen mixtures were obtained from collecting equal numbers of mature anthers from each genotype in a 200 µl PCR tube. Then the tube was vigorously tapped to blend the pollen grains of different genotypes thoroughly. The pollen mixture was applied on the stigma with a sterile toothpick.

In this study, to view the pollen-pollen competition, the traditional mixed pollination was modified by loading the competing pollen from different pollen donors spatially separated on the same stigma. This pollination method was defined as "separated pollination". For separated pollination, the cut on the corolla for emasculation (Fig. 3 and Fig. 9A) was used as a natural mark to track which side of the stigma was pollinated with which pollen. One of the planes of the symmetry of the stigma is the plane that separates the two "transmitting zones" (Fig. 7), and this plane is vertical to the stigmatic groove (cleft) and overlays with the zygomorphic symmetry plane of the corolla. Therefore, the cut on one side of the corolla for emasculation can be used as a mark to distinguish the two sides of the stigma (Fig. 3). In order to randomize

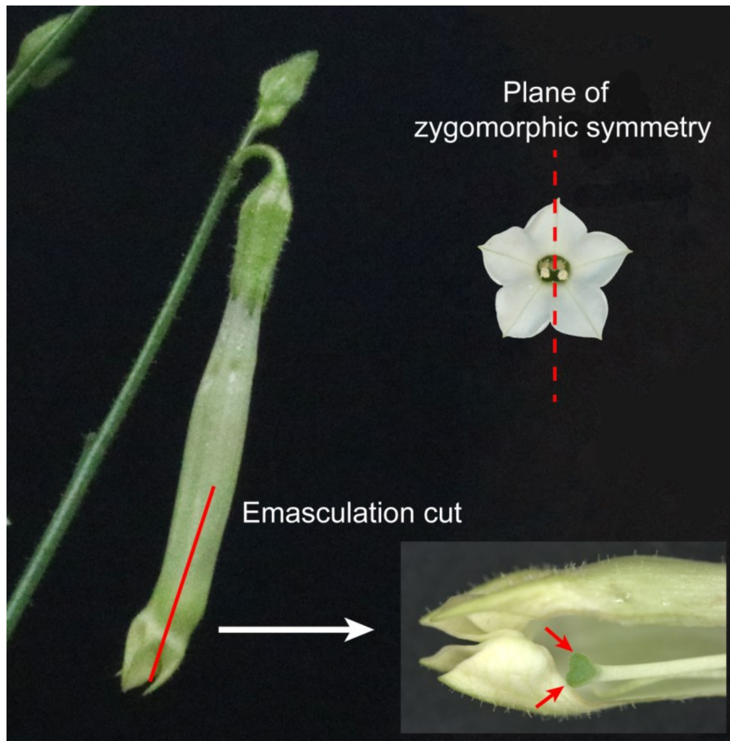


Figure 3. Cutting position of emasculation for separated pollination. The cutting marks one side of the stigma as displayed in the zoomed in picture. One side of the stigma faces to the cut. The cutting was conducted randomly on either side of the corolla. Two red arrows indicate the two stigmatic lobes.

the genotype of pollen loaded on a certain zone of the stigma, the cut on the corolla for emasculation was randomly done on either side of the corolla. Anthers of the different paternal

genotypes was collected separately in individual PCR tubes and mixed in the way described above for the mixed pollination section. The pollen from two tubes was then gently applied on the two zones of the stigma with separate sterile toothpicks, respectively, with a time interval of less than 10 seconds. Non-UtWT pollen was always loaded on the zone of the stigma closer to the cut on the corolla.

3.1.3 Estimation of the number of pollen grains on one zone of the stigma in separated pollination

After putting pollen on one zone of the stigma, the stigma was cut off and fixed (ethanol : acetic acid, 3 : 1) and stained with aniline blue as described in Mori *et al.* (2006). Samples were centrifuged (1000x g, 10 min) to precipitate the pollen grains after each staining and washing step before changing the liquid. Five microliters of each stained sample (40 – 60 µl) was observed under a confocal laser scanning microscope (LSM 880, Zeiss, Germany) in channel mode with a 10x objective (0.45 W C-APOCHROMAT) and a 405 nm laser diode for illumination. Excitation and detection window were set via a 405 nm main beam splitter and a QUASAR detector range between 480 – 550 nm, respectively. Tile scan function was used to obtain the whole area with pollen grains. During the scanning, the pinhole was open completely to include all the signals of the z axis of the sample at once. Later, the pollen was counted manually to estimate the amount of pollen on the stigma.

3.1.4 *In vivo* pollen tube visualization, length measurement and further data acquisition

Styles together with stigmas were harvested without angled cutting at the bottom at 1 h, 4 h, 8 h after pollination. Separate-pollinated styles were harvested with an angled cut at the bottom of the style to distinguish the two zones of the stigma (Fig. 9B). The samples were subsequently fixed and stained with aniline blue as described above. The same confocal laser scanning microscope and the objective described above was used to visualize the PTs in styles which were illuminated by the 405 nm laser diode and the emission light was detected in the 480 – 550 nm detection window of the QUASAR detector. The pinhole size was 71 µm, while the Z-stack step size was 5 µm. Tiled Z stacks were acquired to obtain all the necessary details.

For separate-pollinated samples, a different mounting strategy was used (Fig. 7A) to maintain the 3D structures of the samples. After image acquisition, the scanned tiles were then stitched and the PT lengths were measured in ZEN (black 2012, Zeiss, Germany). Representative images were obtained with maximum intensity projections in ImageJ 1.50e.

For single pollination, samples harvested 4 h and 8 h after pollination showed tangled PTs in the style, and thus only the length of the longest PT was measured. The length was measured from the stigma to the ending position of the longest PT. Since PTs are too short in styles to find the longest one 1 h after pollination (Supplementary Fig. S1B), the length of PT of each style at 1 h time point was a mean value obtained from measuring random 5 visible PTs on a stigma. Samples with less than 10 germinated pollen grains were considered as samples with no germinated pollen (Supplementary Fig. S1A).

In order to observe the differential growth rates (length) of two competitive PTs in the same style after separated pollination, the observing time points needed to be chosen wisely. Bhattacharya and Baldwin (2012) defined the region where the selection on PTs started in *N. attenuata* pistils by using a semi-*in vivo* approach. The selection started at 6 mm in the style apart from the stigma head and it became more prominent at 9 mm. In the single-pollinated styles, the length of the longest PTs reached around 4 mm at the 4 h and 11 mm at the 8 h time point. Therefore, I chose 4 h and 8 h as ideal time points to observe the length difference between two competitive PTs in separated pollinations.

The length difference between two longest PTs of the two competitive pollen donors within the same style was measured in ZEN. Samples with undistinguishable “separated transmitting zones” (Fig. 7) were excluded from the measurement and further analyses. Many samples (especially UtWT styles 8 h after being pollinated with UtWT+G2) had unclear or no gaps between two bundles of PTs, so that the measuring of the length difference between two genotypes of PTs was impossible. The numbers of replicates for each measurement can be consulted in Supplementary Table S1.

3.1.5 Paternity assessment of matured seeds

Paternity was determined from 25 seeds (about 10% of the total seeds per capsule) randomly selected from each capsule. Bhattacharya and Baldwin (2012) demonstrated that 10% of the seeds from each capsule predicted the exact paternity ratio for all seeds within a capsule. The randomly selected seeds were germinated as described above in Section 3.1.1. The seedlings were harvested for DNA extraction and further PCR amplification. The paternity of the seeds obtained from UtWT+G10 pollination combination was determined by PCR-amplifying a region of the trypsin proteinase inhibitor (TPI) gene with the primer pair:

TPI-F: 5’-CTTGTAAGCAATGTGGAACATGCAGATGCC

TPI-R: 5’-TTAGGAAACAGCAACCCTAGACTTCTGGAG.

The seeds sired by G10 showed a band with 180bp deletion compared with the seeds sired by UtWT (Wu *et al.*, 2007 and Guo’s unpublished data; Supplementary Fig. S2). The primers were tested by Guo in different accessions of *N. attenuata* and the results showed stable performance of the primers (unpublished data).

3.1.6 Statistical analysis

Shapiro-Wilk Normality test and Fligner-Killeen test of homogeneity of variances were performed on the *in vivo* PT length data. Subsequently, either Kruskal-Wallis rank sum test or one way ANOVA were done according to the normality and homogeneity of the data set. For data sets with significant main effects, post hoc test (Tukey multiple comparisons of means) was used on the data set that passed the normality and homogeneity tests. Dunn’s test of multiple comparison (1964) was applied on the data set that failed to pass the normality and homogeneity tests. Dunn’s test of multiple comparison was performed by using R package FSA (Ogle, 2017). *P* values were adjusted with the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). Counted data of pollen germination were analyzed with Fisher’s exact test. Binomial generalized linear model (GLM, standard errors were corrected using quasi-GLM models) was performed to analyze the difference between two seed sets obtained from two pollination methods. Chi-square goodness-of-fit test was used to test the significance of

nonrandom mating with the given ratio of the paternity 1 : 1. All the statistical analyses mentioned above were performed in R (RCoreTeam, 2016).

3.1.7 Metabolomics analyses of pollinated styles

For metabolomics analyses, UtWT styles pollinated with UtWT+G2 and UtWT+G10 pollen combinations, and irACO styles with UtWT+G10 pollination combination were chosen to identify potential metabolites involved in mate selection. Five styles with stigmas were sampled as one replicate 8 h after pollination in a 1.5 ml Safe-Lock Eppendorf tube. They were kept in liquid nitrogen until they were stored at -80 °C. Five replicates of each treatment were sampled. The styles were ground for 1 minute with two metal beads (Ø 2 mm) at 1000 strokes min^{-1} in Geno/Grinder 2000 (SPEX SamplePrep). Then, 150 µl ice-cold 80% MeOH was added to each sample to extract the metabolites. Tubes were stored overnight at -20 °C. The tubes were centrifuged (15000x g, 20 min, 4 °C) and the supernatant was transferred to a fresh 1.5 ml Eppendorf tube. The extracts were centrifuged again following the same method and the supernatant from each sample was transferred to glass vials with inserts.

The extracts were analyzed with ultrahigh-performance liquid chromatography-electrospray ionization/ high resolution quadrupole time-of-flight mass spectrometry (UHPLC-ESI/HR-Q-TOF-MS). One microliter of each extract was injected into a Dionex Ultimate 3000 UHPLC system (Thermo Scientific, Dionex, Sunnyvale, California, USA). An Acclaim RSLC 120 C18 column (150×2.1 mm, particle size 2.2 µm, average pore diameter 120 Å, Acclaim, Dionex, Sunnyvale, USA) was used to separate the metabolites. The mobile phase was composed of two types of solvent. Solvent A consisted of deionized water containing 0.1% (v/v) acetonitrile and 0.05% (v/v) formic acid. Solvent B was composed of acetonitrile with 0.05% (v/v) formic acid. The following binary gradient was used: 0 to 3 min, isocratic 10% B; 3 to 12 min, linear gradient to 20% B; 12 to 17 min, linear gradient to 35% B; 17 to 23 min, linear gradient to 40% B; 23 to 25 min, linear gradient to 45% B; 25 to 30 min, linear gradient to 50% B; 30 to 40 min, linear gradient to 98% B; 40 to 45 min, at 98% B. Flow rate was 400 $µl \cdot min^{-1}$.

Eluted compounds were detected by a high-resolution Q-TOF mass spectrometer (Brucker Impact II, Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization source operating in positive ion mode. Typical instrument settings were as follows: end plate offset, 500 V, capillary voltage, 4500 V; nebulizer pressure, 1.4 bar; dry gas temperature, 200 °C; dry gas flow, 10 L·min^{-1}. Ions were detected over a range of *m/z* 50 to 1500. Mass calibration was performed using a direct infusion of sodium formiate calibration solution (50 ml isopropanol, 200 µl formic acid, 1 ml 1 M NaOH in water). Raw data files were calibrated by using the Bruker high-precision calibration algorithm and converted to netCDF format using the export function of the Data Analysis v4.0 software (Bruker Daltonics, Bremen, Germany).

The files were processed in R (RCoreTeam, 2016) using the XCMS package (Smith *et al.*, 2006; Tautenhahn *et al.*, 2008). Peak detection was performed by the centWave method (Tautenhahn *et al.*, 2008) with the parameter settings ppm = 10, snthresh = 10, peakwidth = 5 s to 18 s. Peak grouping and retention time correction were achieved via the parameter settings minfrac = 0.8, bw = 10 s, mzwid = 0.01 D, and family = “s”, missing = 1, extra = 1. The fillPeaks routine of the XCMS package was applied to estimate the missing features, and then the data matrix was written into Microsoft Excel for further statistical analysis. Only mass features with an intensity of > 1500 in at least 80% of the 5 replicates of at least one treatment were considered for further analyses. Annotation of isotope, adduct and fragment peaks and clustering of mass features into “compound spectra” was performed with CAMERA package (Kuhl *et al.*, 2012) in R after XCMS data processing. Based on the ion species annotation, +1, +2 and +3 isotopic peaks were excluded to reduce information redundancy. The missing values (zeros) were imputed with k-means nearest neighbor (kNN) method in R with VIM package (Templ *et al.*, 2013), since Armitage *et al.* (2015) suggested that metabolomics data imputed with kNN method showed the best restoring of normality and homogeneity of variance of the data. The imputed data was normalized by applying 75th percentile normalization. After normalization, data was log2 transformed for further analyses. The transformed data were imported into MeV 4.9.0 (Saeed *et al.*, 2003) for pairwise t-test comparisons.

3.1.8 Metabolite analyses on non-pollinated styles and *in vitro* PTs

After emasculation, the non-pollinated UtWT flowers were left on the plant for 8 h, and then each unpollinated style was sampled as one replicate in a 1.5 ml Eppendorf tube. After being ground, 100 µl ice-cold 80% MeOH was added to each sample. The following extraction procedures were identical as described above in Section 3.1.7. In total 10 replicates were harvested and analyzed.

Five anthers from UtWT and 5 anthers from G10 were collected from fully opened flowers and mixed together in an Eppendorf tube. Due to the static electricity, pollen grains were attached to the wall of the tube, and thus the remaining parts of anthers could be removed by inverting the tube. Five hundred microliters of freshly prepared liquid PT growth medium (15 mM MES pH 5.9, 1.0 mM $CaCl_2$, 1.0 mM KCl, 0.8 mM $MgSO_4$, 1.6 mM H_3BO_3, 0.01 mM $CuSO_4$, 10% sucrose, 0.03% casein hydrolysate) was added into the tube. Then the tube was incubated in a growth chamber (Percival, Perry Iowa, USA) at 26 ˚C, dark for 8 h. Five biological replicates were collected and cultured in the same way.

After incubation, PTs were centrifuged down at 1000x g for 1 min. Subsequently, 480 µl of the medium was removed. Samples then were extracted with 200 µl 80% MeOH. The samples were incubated in an ultrasonic bath at room temperature for 5 min. Samples were centrifuged twice (15000x g, 20 min, 4 °C) and the supernatants were transferred to Eppendorf tubes. Since the extracts consisted of high concentration of sucrose (from the medium), they were not suitable for highly sensitive MS instruments. Therefore, to remove high abundant constituent of the PT growth medium from the extracts, samples were purified by reversed phase solid phase extraction using a 96-well HR-X column (MACHEREY-NAGEL, 96 × 25 mg, catalog number: 738530.025M) and a Chromabond Multi 96 vacuum manifold (MACHEREY-NAGEL, catalog number: 738630.M). The columns were activated first with 600 µl (per well) MeOH and then conditioned with 600 µl H_2O. Before loading the samples on the columns, the MeOH concentration of the extracts was lowered by diluting them in 2 ml of H_2O. The samples were loaded stepwise on the columns and the flow through was discarded. After washing the

columns twice with 1 ml H_2O the samples were eluted with 500 µl 100% MeOH. In order to get better signals on the MS, the injection volume into the UHPLC-ESI/HR-Q-TOF-MS was increased to 5 µl.

The extracts of styles and PTs were analyzed with the same equipment and mass spectrometer set up described in Section 3.1.7. The only difference was the binary gradient used in LC method. The solvent gradient used here was: 0 to 0.5 min, isocratic 10% B; 0.5 to 23.5 min, linear gradient to 90% B; 23.5 to 25 min, isocratic 90% B. Raw data files were calibrated by using the Bruker high-precision calibration algorithm and converted to netCDF format using the export function of the Data Analysis v4.0 software and then the presence of certain *m/z* values selected by the analyses mentioned in Section 3.1.7 were manually checked in Data Analysis v4.0 software.

3.2 Paternal effects on seed metabolomes and differential pathogen resistance

3.2.1 Plant materials and hand pollination for hybrid seeds

From Utah, 11 different lines were collected from native populations (Fig. 4) between 1993 and 2009 (G1 to G11, see descriptions in Schuman *et al.*, 2009), and then screened and selfed by Bhattacharya (unpublished data). G2 and G10 used here are the same genotypes used in the PT competition experiments described above. These 11 genotypes were used as pollen donors in hand pollinations to get hybrid seeds on the 15th generation of UtWT plants (described above in Section 3.1.1). Fifteen independent collections of seeds were obtained from different natural plants and populations in Arizona (Fig. 4) between 1995 and 2003. The first inbred generation of these 15 collections were attained in the greenhouse by selfing. These 15 genotypes were used as different pollen donors to generate hybrid seeds from the 7th generation of AzWT plants, which was originally collected in 1996 from a population near Flagstaff, Arizona, USA (Glawe *et al.*, 2003).

Seeds were germinated as described in Krügel *et al.* (2002). Plants were grown under uniform conditions as described in Section 3.1.1. Maternal flowers were emasculated by

applying the protocol described above in Section 3.1.2. After emasculation, each maternal stigma was loaded with a single genotype of pollen from a pollen donor by rolling anthers on the stigma. Seeds from different capsules with the same parentage were collected and pooled together in a vial. Seeds were dried completely in a desiccator and stored at room temperature for later usage.

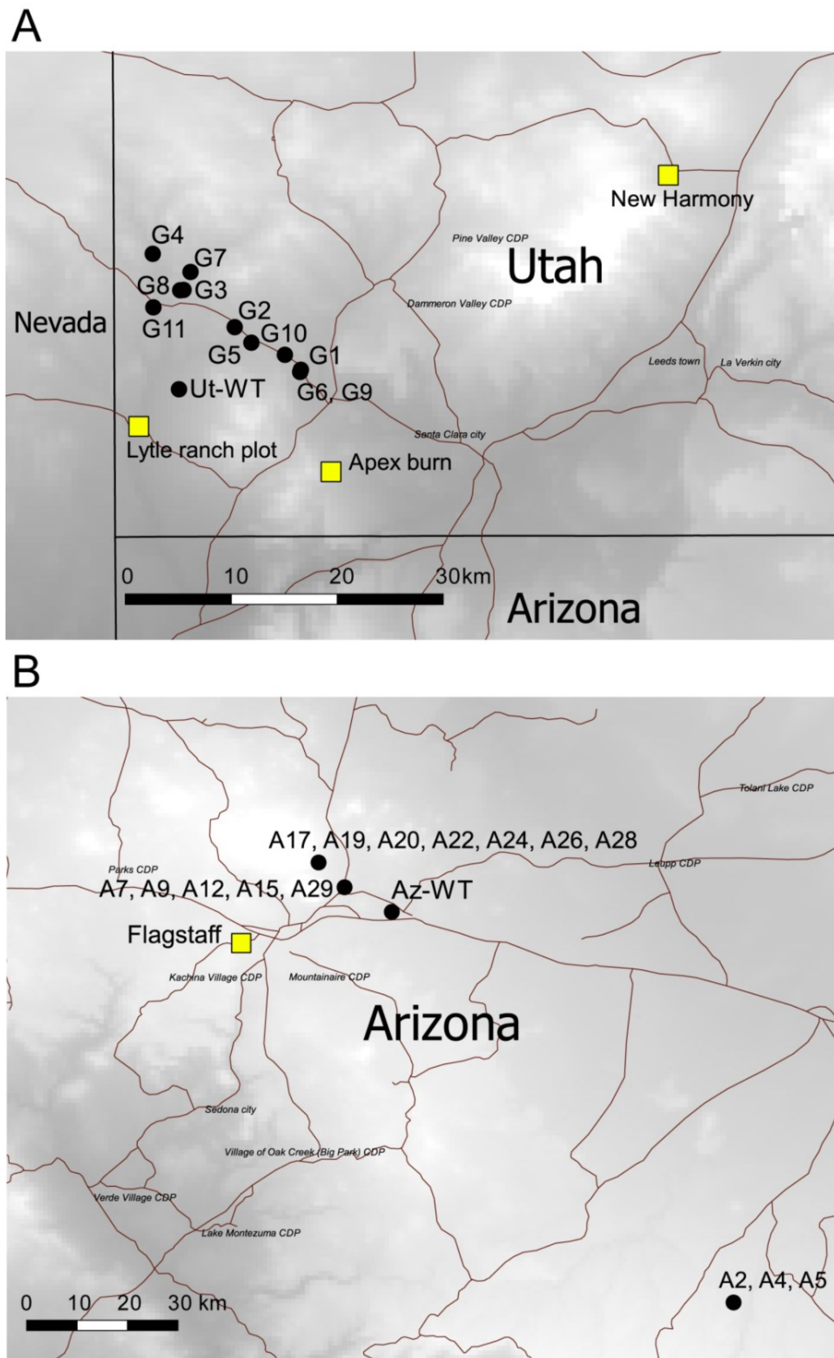


Figure 4 The distributions of the collection sites of the native lines used in this study and the locations of the seed burial sites. A) Lines collected from Utah (black dots) and the seed burial sites in Utah (yellow squares). B) Lines collected from Arizona (black dots) and the seed burial sites in Arizona (yellow squares).

3.2.2 Seed metabolite extraction and further metabolomics analyses

Ten milligrams of dry *N. attenuata* seeds of each of the 26 genotypes were weighed as one replicate. Five replicates for each genotype of seeds were used. One milliliter sterile deionized water was added into each Eppendorf tube, and the seeds were imbibed for 1 day at room temperature. After removing the water, seeds were fast frozen in liquid nitrogen and ground thoroughly. Metabolites were extracted from the ground seeds by adding 1.5 ml 50% MeOH and being incubated at room temperature for 1 day. Subsequently, 20 min ultrasonic bath was applied on the samples. The extracts were centrifuged at 16,000 g for 30 min twice to remove all the particles. The supernatants were transferred in glass vials for further analyses.

The metabolites were separated and analyzed by applying an ultrahigh-performance liquid chromatography-electrospray ionization/time-of-flight mass spectrometry (UHPLC-ESI/TOF-MS). Two microliters of each sample were injected into a Dionex Ultimate 3000 UHPLC system (Thermo Scientific, Dionex, USA). A same column and mobile phase described in Section 3.1.7 were used to separate the metabolites. The following binary gradient was used: 0 to 0.5 min, isocratic at 10% B; 0.5 to 13.5 min, linear gradient to 80% B; 13.5 to 14 min, linear gradient to 100% B; 14 to 15 min, isocratic at 100% B. Flow rate was 400 $\mu l \cdot min^{-1}$.

Eluted compounds were detected by a TOF mass spectrometer (Brucker MicroTOF, Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization source operating in positive ion mode. The instrument was set as follows: capillary voltage, 4500 V; nebulizer pressure, 1.2 bar; dry gas temperature, 200 °C; dry gas flow, 8 $L \cdot min^{-1}$. Ions were detected over a range from 50 to 1400 *m/z*. Mass calibration was conducted by using the same solution mentioned in Section 3.1.7. Raw data files were calibrated and converted to netCDF format by applying the same method described in Section 3.1.7.

The raw data files were grouped according to the seed maternities and were processed separately. The files were processed in R (RCoreTeam, 2016) using the XCMS package (Smith *et al.*, 2006; Tautenhahn *et al.*, 2008). Peak detection was performed by the centWave method

(Tautenhahn *et al.*, 2008) with the parameter settings ppm = 20, snthresh = 30, peakwidth = 5 to 10 s. Peak grouping and retention time correction were achieved via the parameter settings minfrac = 0.8, bw = 5 s, mzwid = 0.01 D, and family = "s", missing = 1, extra = 1. The fillPeaks routine of the XCMS package was applied to estimate the missing features. Zeros were inserted for the missing values at this stage. Mass features with an intensity > 1500 in at least 80% of the 5 replicates of at least one seed genotype were considered for further analyses. Annotation of isotope, adduct and fragment peaks and clustering of mass features into "compound spectra" was performed with CAMERA package (Kuhl *et al.*, 2012) in R after XCMS data processing. Based on the annotation, +1, +2 and +3 isotopic peaks were excluded from further analyses to reduce information redundancy. The refined data matrices were imported into the web-based software MetaboAnalyst 3.0 (Xia *et al.*, 2009; Xia *et al.*, 2015) for principal component analysis (PCA). The zeros were imputed with the half of the minimum positive values in the imported data sheet to reduce the presence of zeros. Subsequently, the data was normalized by applying sample median normalization. After normalization, data was auto scaled by being mean-centered and divided by standard deviation of each variable (feature) for further PCA. All the missing-value imputation, data normalization and scaling were calculated in MetaboAnalyst 3.0. The normalized and scaled intensities of the potentially interesting features from PCA were then imported into MetaboAnalyst 3.0 for hierarchical cluster analysis and generating the heatmap. Euclidean distance and Ward's linkage were applied to compute the dendrogram. The tentative feature identities were assigned by searching *m/z* values in an eFP Browser-based *N. attenuata* online database (http://nadh.ice.mpg.de/NaDH/).

3.2.3 Fungus isolation and culturing

In order to investigate potential fungal pathogens seeds may encounter in their native habitats, seeds buried in field (both Utah and Arizona, Fig. 4) for a year were germinated on Gamborg's B5 medium (GB5, Duchefa, The Netherlands) petri dishes in the lab by using the germination method described above in Section 3.1.1 with slight modifications. Seeds retrieved from the field were washed in water to remove the soil and sand. After that, seeds were incubated in sterilizing solution (2% dichloroisocyanuric acid and 0.005% Tween 20) for 1 h

instead of 7 min described in Krügel *et al.* (2002). Seeds were, then, washed twice with water to remove the sterilizing solution. Subsequently, the seeds were incubated in liquid smoke supplemented with GA_3 (1 mM) overnight instead of 1 h described in Krügel *et al.* (2002). After incubation, seeds were washed once and placed on GB5 plates. The GB5 plates were maintained in a growth chamber (Percival, USA) at 26 °C with 16 h light and at 24 °C with 8 h darkness. Fungal isolates were obtained both from germinated and dead seeds (14 days after the seeds were placed on the GB5 petri dishes) by transferring a small piece of mycelium surrounding the seed onto a potato dextrose agar (PDA, Sigma, Germany) petri dish with 100 $\mu g \cdot ml^{-1}$ of streptomycine sulfate (Sigma, Germany). According to morphologies, fungal colonies were chosen from GB5 plates and purified on PDA plates. The PDA plates with fungus colonies were kept at 25 °C in dark. Only one of the colonies showing the same morphology was isolated, purified and stored for further usage. Purified colonies were later transferred on PDA slants. After 3 days of incubation at 25 °C, in dark, on PDA slants, the obtained fungal colonies with slants were stored at 4 °C to preserve them for long period.

3.2.4 Fungal DNA extraction and identification

Fungal mycelia were harvested from 14-days-old fungal cultures in the manner described by Schuck *et al.* (2014). Frozen mycelia were manually ground in liquid nitrogen. Fungal DNA was extracted by applying the cetyltrimethylammonium bromide (CTAB) method according to Bubner *et al.* (2004) and Schuck *et al.* (2014).

Fungi were identified molecularly by sequencing the internal transcribed spacer (ITS) regions. Primer pairs ITS1-F and TW13 or ITS1-F and ITS4 were used to obtain the target fragments (White *et al.*, 1990; Gardes *et al.*, 1991; Gardes and Bruns, 1993; O'Donnell, 1993). A 600 – 700 bp long DNA sequence consisting of the ITS1 and ITS2 regions, the 5.8S rDNA and a small part of the large ribosomal subunit (LSU) gene was amplified by PCR on fungal DNA samples by using the primer pair ITS1-F (5'-TTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). For fungal DNA samples which showed no amplification products with this primer pair, an alternative primer pair (ITS1-F and TW13: 5'-GGTCCGTGTTTCAAGACG-

3') was applied to obtain an approximately 1200 bp DNA fragment comprising ITS1 and ITS2 regions, the 5.8S rDNA and a longer part of the LSU gene. PCR products were obtained from the PCR program with denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 54 °C for 20 s and elongation at 72 °C for 1 min, followed by a final extension step at 72 °C for 5 min. High-fidelity polymerase (Phusion polymerase with manufactory buffer) was used for PCR reactions.

Direct sequencing was performed according to Schuck *et al.* (2014). All sequences were assembled and edited in Geneious 6.0.5 (Biomatters Ltd, New Zealand). The final sequences were compared to sequences in the GenBank database by applying BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/) to obtain species identities as described in Schuck *et al.* (2014). Utah and Arizona showed quite distinct fungal species. In this study, I focused on 3 fungal species (*Alternaria* sp., *Fusarium acuminatum* and *Trichoderma harzianum*, see Supplementary Table S2 for ITS sequences) isolated from Arizona. Many species from *Alternaria* genus are known as plant pathogens (Thomma, 2003; Kumar *et al.*, 2014). *F. acuminatum* is considered as one of the primary causal agents of root rot of wheat (Burgess *et al.*, 1975; Hill *et al.*, 1983; Mergoum *et al.*, 1998). Furthermore, some studies showed that *F. acuminatum* was a weak pathogen for several plant species (Fernandez *et al.*, 1985; Axelrood *et al.*, 1995). Therefore, these two fungal isolates were selected for further pathogenicity tests on *N. attenuata* seeds. Since *T. harzianum* is a well-known biocontrol agent and functions as a parasite or antagonist of many phytopathogenic fungi (Vinale *et al.*, 2008), it was included in this study to investigate if the growth of the pathogens of *N. attenuata* seeds can be inhibited when it is present.

3.2.5 Fungal morphology observation

The selected 3 fungal species (*Alternaria* sp., *F. acuminatum* and *T. harzianum*) were observed under a light microscope to capture the characteristic structures of their conidia and conidiophores. A piece of mycelium was picked out from a 10 – 14-days-old colony of *Alternaria* sp., *F. acuminatum* or *T. harzianum* cultured on PDA and spread apart in tubes of water,

respectively. Mycelia were viewed under the Leica LMD6000 microscope (Leica Microsystems, Wetzlar, Germany) with 40x or 63x objective in transmitted light bright field set-up. As for *F. acuminatum*, only microconidia were observed from the colony cultured on PDA. In order to observe the macroconidia, mycelium was obtained from infected *N. attenuata* seeds placed on GB5 medium for 10 days. The inoculation process and germination process of the seeds are described below in Section 3.2.6. The imbibed seeds without sterilization and inoculation were kept on PDA for 7 days, and were then sterilized and germinated on GB5 as controls, to confirm that the fungal colony emerging from the inoculated seeds was the inoculum. The absence of emerging fungal or bacterial colonies (both on PDA and GB5 medium) on the control seeds suggested that the seeds used in this research were sterile.

3.2.6 Pathogen bioassays and dual culture assays

In order to demonstrate the impact of paternal plants on the resistance of their offspring seeds to fungal pathogens, hybrid seeds with different paternities that showed divergence in the PCA of seed metabolomic profiles were selected for the bioassays. The fungal species chosen for pathogenicity tests were all isolated from Arizona. To investigate the interaction between seeds and the native microbes, the bioassays were focused only on the hybrid seeds with Arizona genetic background. A 6 mm mycelial plug was placed on the center of each PDA plate and maintained at 25 °C in darkness. Seeds of different genotypes were washed in sterile water twice, and then imbibed in water for 1 h. After 3-days culturing on PDA, around 20 imbibed seeds were placed on the edge of each fungal colony (Fig. 5). Imbibed seeds placed on sterile PDA plates were used as controls. For each combination of fungus and seed genotype, 5 independent PDA plates were used as replicates. The plates were incubated at 25 °C in the dark for 7 days. After incubation, seeds were picked out from each plate and washed with sterile water. Subsequently, seeds were germinated as described above in Section 3.2.3 with a tiny modification. Seeds were incubated in sterilizing solution for 2 h instead of 1 h. After 10 days of germination, the germination rate was recorded. Seeds with visible radicles were considered as germinated and alive seeds.

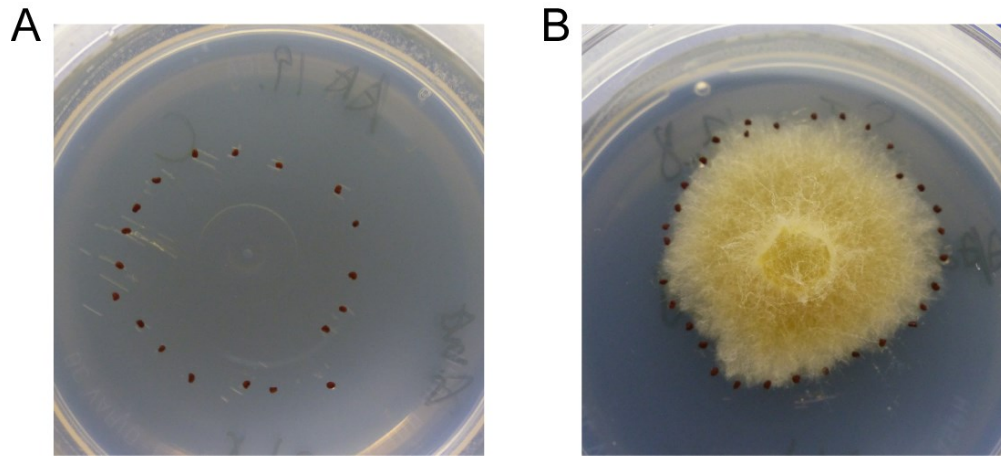


Figure 5. Representative pictures of the bioassay method used for pathogenicity test. A) Representative picture of the control of the bioassay. Imbibed seeds were placed on the potato dextrose agar (PDA) medium without sterilization. B) Representative picture of the fungal treatment of the bioassay. Imbibed seeds, without sterilization, were placed on the edge of the fungal colony growing on PDA.

To investigate the antagonistic activity of *T. harzianum* against the fungal pathogens, a 6 mm mycelial plug of *T. harzianum* was placed on one half of a PDA plate, while a same-sized mycelial plug of the pathogen was placed on the other half. A PDA plate with two mycelial plugs of the same fungus placed on each half of the plate was set as a control. The radial growth of the fungus (C and T) was measured after 3 and 6 days. C is radial growth of the pathogenic fungus in control; T is radial growth of the pathogenic fungus in the presence of *T. harzianum*. For each replicate, C or T was an average value obtained from measuring about 5 times of the distance between the edge of the plug and the edge of the colony. Each time of the measurement was done at different positions of the colony. Percent inhibition of average radial mycelial growth of each fungal pathogen was calculated in relation to the growth of the controls according to the following equation:

$$L = [(C - T) / C] \times 100\%$$

Where L is inhibition of radial mycelial growth.

The interaction areas (or inhibition zones) between *T. harzianum* and pathogenic fungi were viewed under a stereomicroscope (AXIO Zoom.V16, Zeiss, Germany) 3 days after dual culture. Example images of mycoparasitism and inhibition zone were obtained with the

stereomicroscope (AXIO Zoom.V16, Zeiss, Germany) equipped with a Plan-Neofluar Z 1.0x objective and a CCD camera (Axiocam 506 color) in reflected light bright field mode. To observe detailed morphologies, Z-stack scanning was performed to obtain all the necessary details. The acquired Z stacks were used in Helicon Focus 6.3.7 Pro (www.heliconsoft.com) to generate an extended focus image by applying pyramid method.

3.2.7 Statistical analyses

Counted data of germinated and dead seeds in bioassays were analyzed by applying binomial generalized linear model (GLM). Since the data exhibit overdispersion, quasi-likelihood was used to specify the variance function in a GLM. Quasi-binomial GLM was performed to analyze the difference between data from pairwise comparisons of seed genotypes treated with the same fungus. The Fisher's exact test was applied to each genotype of seeds to detect germination reduction when seeds were infected with a certain fungus. If the fungus significantly reduced the germination rate of any genotype of the seeds, the fungus was considered as a pathogen of *N. attenuata* seeds. The statistical analyses were performed in R (RCoreTeam, 2016).

4. Results

4.1 *In vivo* pollen tube competition and secondary metabolites implicated in nonrandom mating of *N. attenuata*

4.1.1 Pollen performance showed no significant difference among single pollinations

I first evaluated the pollen performance in UtWT styles when single pollinations were conducted (Fig. 6). The percentages of pistils with germinated pollen indicated no significant difference (Fisher's exact test; P = 0.1969, P = 0.6793, P = 0.5928, respectively) among UtWT, G2 and G10 single pollinations at 1 h, 4 h and 8 h time points of measurement. The data sets of the length of the PT *in vivo* at 1 h and 4 h time points passed the Shapiro-Wilk Normality test (W = 0.964, P = 0.6225; W = 0.964, P = 0.2189, respectively) and Fligner-Killeen test of homogeneity of variances (χ^2 = 0.993, df = 2, P = 0.6086; χ^2 = 0.822, df = 2, P = 0.6628, respectively). The following ANOVA test showed no significant difference of the length of the PT *in vivo* among single pollinations (at 1 h, $F_{2,17}$ = 0.429, P = 0.658; at 4 h, $F_{2,38}$ = 0.482; P = 0.621). Since the data set of the length of the PT at 8 h failed to pass the Shapiro-Wilk Normality test (W = 0.923, P < 0.001) and Fligner-Killeen test of homogeneity of variances (χ^2 = 8.824, df = 2, P < 0.05), Kruskal-Wallis rank sum test was applied to estimate the difference of the length of PT among pollinations. There was no significant difference among single pollinations at 8 h time point (H = 2.9189, df = 2, P = 0.232). Hence, there was no significant difference of pollen germination and PT growth rate in UtWT styles among single pollinations.

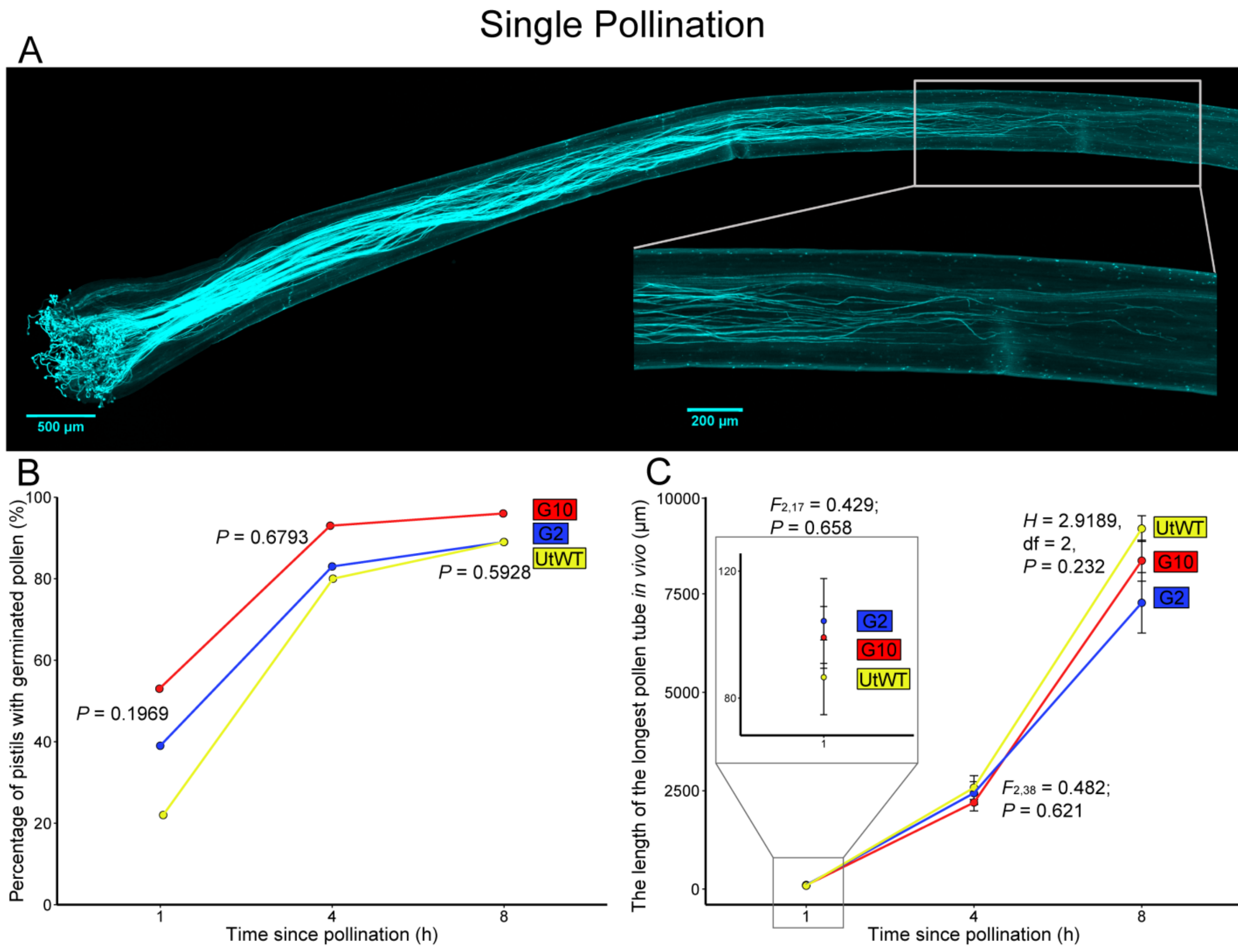


Figure 6. No significant difference in pollen germination and growth kinetics of PTs in UtWT style from single pollinations with natural pollen genotypes. A) Representative maximum intensity projected image of PT growth in an emasculated UtWT flower harvested 8 h after being pollinated with UtWT pollen. As individual PTs could not be separately measured during their growth in the style after 4 and 8 h of pollination, length of the longest PT was measured from the stigma to the tip of the PT. Since PTs are too short to pick out the longest one 1 h after pollination (Supplementary Fig. S1), the mean length of 5 randomly selected PTs of each style was obtained as the length of the longest PTs. B) After 1, 4, and 8 h of single pollinations of emasculated UtWT flowers with UtWT, G10 or G2 pollen, the complete styles (stigma and style) were harvested at the junction of the style and the ovary. The three time points enclose the optimal window of visualization of the PT competition before they reach the ovary. Germination efficiency of all pollen genotypes on stigma were similar (Fisher's exact test) at the three time points of measurements. C) The length of the longest PT *in vivo* showed no significant differences (ANOVA test or Kruskal-Wallis rank sum test) among pollen donors at any of the three time points.

4.1.2 The novel sample mounting method and the description of the two transmitting zones

In order to observe more details and keep the 3D structure of the sample, a new sample mounting strategy was developed and applied (Fig. 7A). During sample mounting, stained styles tend to orient in the anterior position (Fig. 7B). When the stained styles were rotated by 90°, in some samples a clear gap between two bundles of PTs and beneath the vascular bundle can be observed (Fig. 7C). A *N. attenuata* pistil has two fused carpels, and a stigma has two distinguishable lobes (Fig. 7B). Each carpel has a vascular bundle traversing the stigma and the style in the peripheral cortex. This gap is perpendicular to the cleft that separates the two lobes of the stigma. The two separated bundles of PTs indicate two halves of the stigma, which are perpendicular to the stigmatic groove, too. If the style was pollinated on one half of the stigma, PTs were limited in one half of the style 4 h after pollination (Fig. 7D). The two halves of the stigma were defined as “two zones” of the stigma and the two parts of the transmitting tissue in the style were defined as “two transmitting zones” (Fig. 7E).

The mixed-pollination method that applies pollen mixture on the stigma makes it impossible to track the PT genotypes *in vivo* later by staining the PTs and viewing them under microscopes. The discovery of the two transmitting zones and the developing of the new mounting method provided a potential way to track the PT genotypes *in vivo*, if the mixed pollination is done by separating the two genotypes of pollen and loading them separately on the two zones of the stigma, and if this new pollination method does not change the seed set. This structure was leveraged in our later research and the new mixed pollination was defined as “separated pollination”.

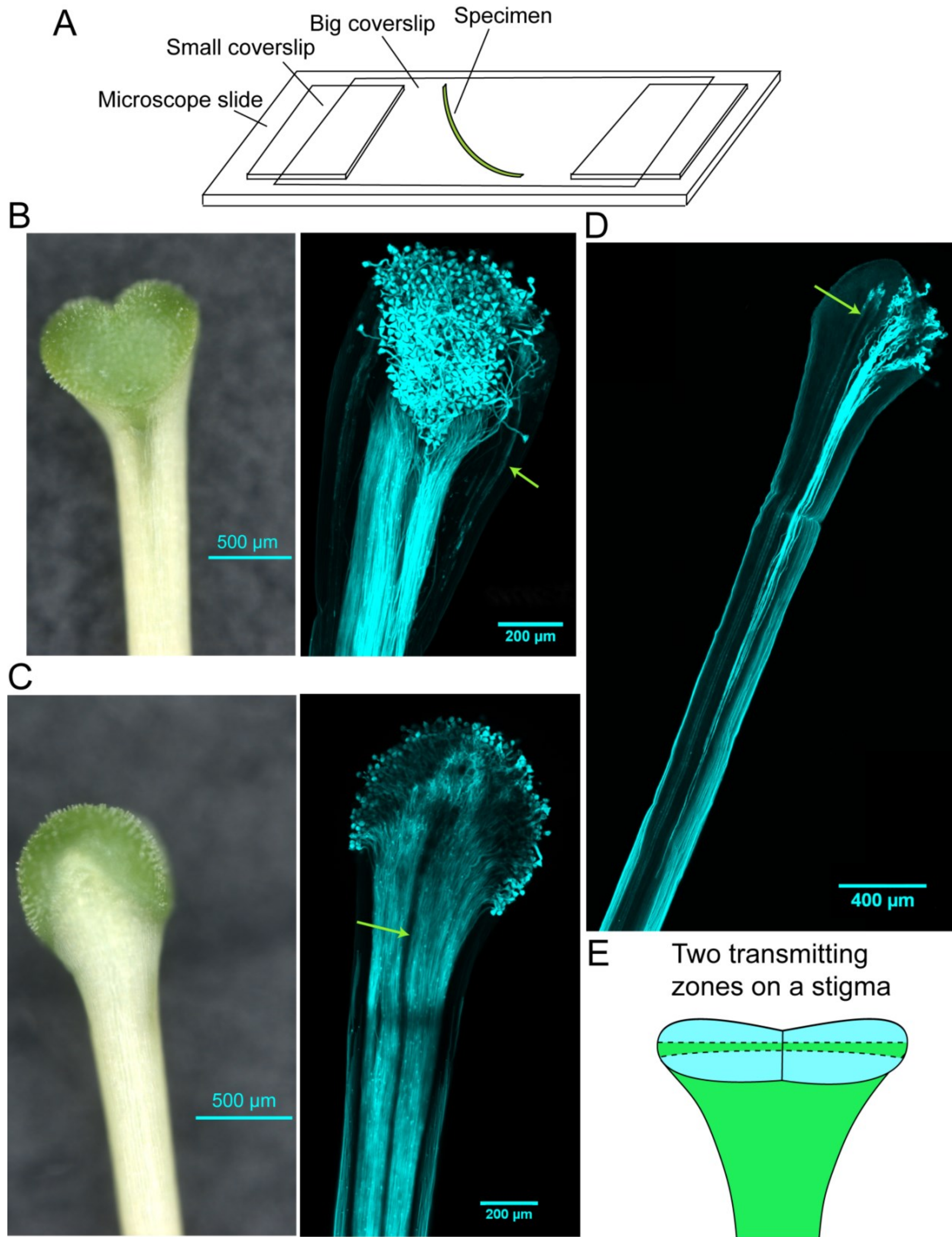


Figure 7. PTs grew separately in parallel transmitting zones, separated by the vascular bundle, in the style. A) Depiction of a novel sample mounting strategy to maintain the 3D structure of the pollinated samples. The space created by the small coverslips protected the samples from being crushed by the big coverslip. B) Anterior view of a non-pollinated style and an aniline blue stained style 8 h after pollination. The arrow indicates the vascular bundle. C) Lateral view of a non-pollinated style and an aniline blue stained style 8 h after pollination. Separate

“two tracks” of PTs can be seen from the lateral view of the stained style. The arrow refers to the gap between two transmitting zones corresponding to the vascular bundle. D) The lateral view of a stained style 4 h after pollination on one transmitting zone of the stigma. PT growth was limited within one zone, while the other half of the style shows no cross over of PTs. The arrow points at the vascular bundle. E) Graphical representation of a stigma with two zones (marked with dash lines) for the placement of pollen. All microscopy pictures were obtained from UtWT styles pollinated with UtWT pollen.

4.1.3 Two different mixed-pollination methods led to the same seed sets

I performed mixed pollination and separated pollination (UtWT+G10) on emasculated UtWT flowers. The seed sets obtained from two different pollination methods showed no significant difference (Fig. 8, Quasibinomial GLM, df = 13, t = 0.502, P = 0.625). It suggested that the change of pollination method did not affect the nonrandom mating significantly. However, although G10 consistently sired less seeds than UtWT, each method showed big variance among replicates (Supplymentary Fig. S2C), which suggested that flowers were different from each other.

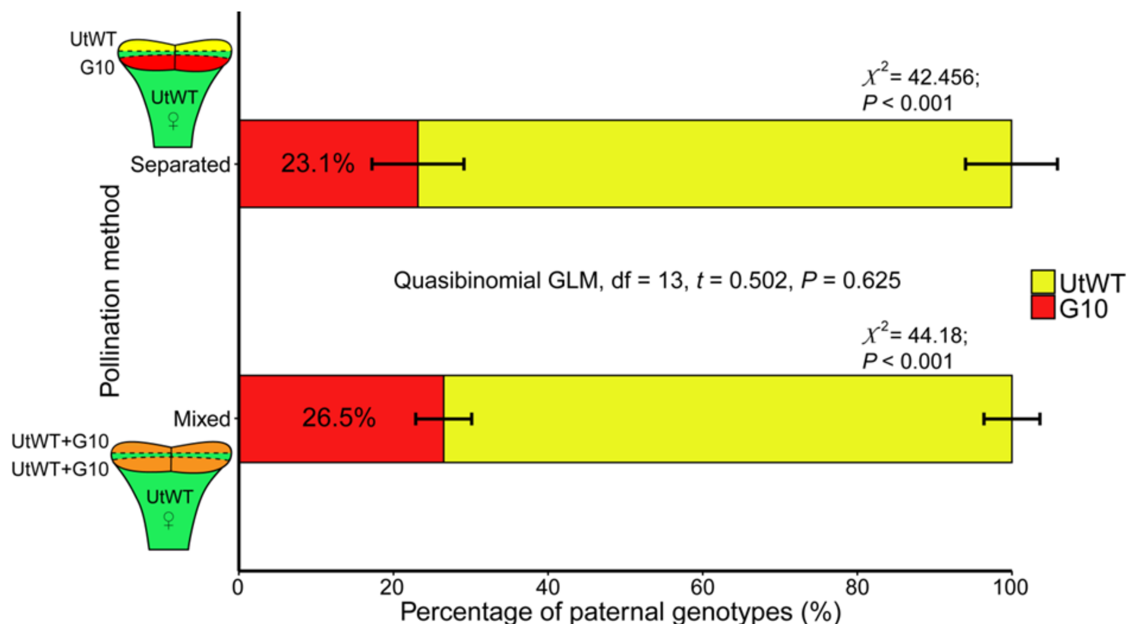


Figure 8. No difference between seed sets from the capsules obtained from different pollination methods. Pollination method in which the pollen mixture, containing mix genotype of pollen, was applied on both zones of stigmas was defined as traditional mixed-pollination (mixed) method. Pollination method in which two different pollen mixtures, each of them containing pollen from a single genotype, were applied separately on two zones of

stigmas was defined as separated two-zones pollination (separated) method. Binomial generalized linear model (GLM, standard errors were corrected using quasi-GLM models) was performed to analyse the difference between two seed sets obtained from two pollination methods. Chi-square goodness-of-fit test was used to test the significance of nonrandom mating, where the assumed random mating ratio of the binary paternity of the seed set is 1 : 1.

4.1.4 The differential *in vivo* PT growths of two pollen donors were involved in disequilibrium paternity

The emasculation mark was sufficient to track the position of the two competitive pollen donors on the stigma (Fig. 9A). The angled cutting, marked at the bottom of the style during sample collection, was used as a label to distinguish the two transmitting zones under the microscope (Fig. 9B), by doing this, the genotypes of the PTs can be distinguished. At 4 h and 8 h, the length of non-self PT (G2 or G10) was constantly shorter than UtWT PT in UtWT styles. At the same time points, the length of non-self PT (G10) was shorter, equal or longer than UtWT PT in non-selective (irACO and ETR1) styles. The length difference between the longest PT of one pollen donor and the longest PT of the other pollen donor within the same style was measured. If G2 or G10 PTs were shorter than UtWT PTs, the difference between PTs of two donors was recorded as a positive value. On the contrary, if G2 or G10 PTs were longer than UtWT PTs, the difference between PTs of two donors was recorded as a negative value.

The data set of the length difference of PTs at 4 h time point passed the Shapiro-Wilk Normality test (W = 0.971, P = 0.4153) and Fligner-Killeen test of homogeneity of variances (χ^2 = 2.279, df = 3, P = 0.5165). The following ANOVA and post hoc test showed the length difference between UtWT and G10 (non-favored non-self pollen donor on UtWT styles) PTs in UtWT styles was significantly bigger than the differences in non-selective maternal styles (irACO and ETR1) ($F_{3,34}$ = 6.602; P < 0.01). However, the length difference between UtWT and G2 (favored non-self pollen donor on UtWT styles) PTs in UtWT styles had no significant difference compared with the length difference between UtWT and G10 PTs in UtWT styles and non-selective styles. In addition, at 4 h, all the samples checked showed clear gaps between the two bundles of PTs, with only one exception (Fig. S3 and Table S1). Because the PT identities in the sample with

unclear gap between the two PT bundles of the two pollen donors could not be distinguished, the length difference between the two PT bundles was not measured.

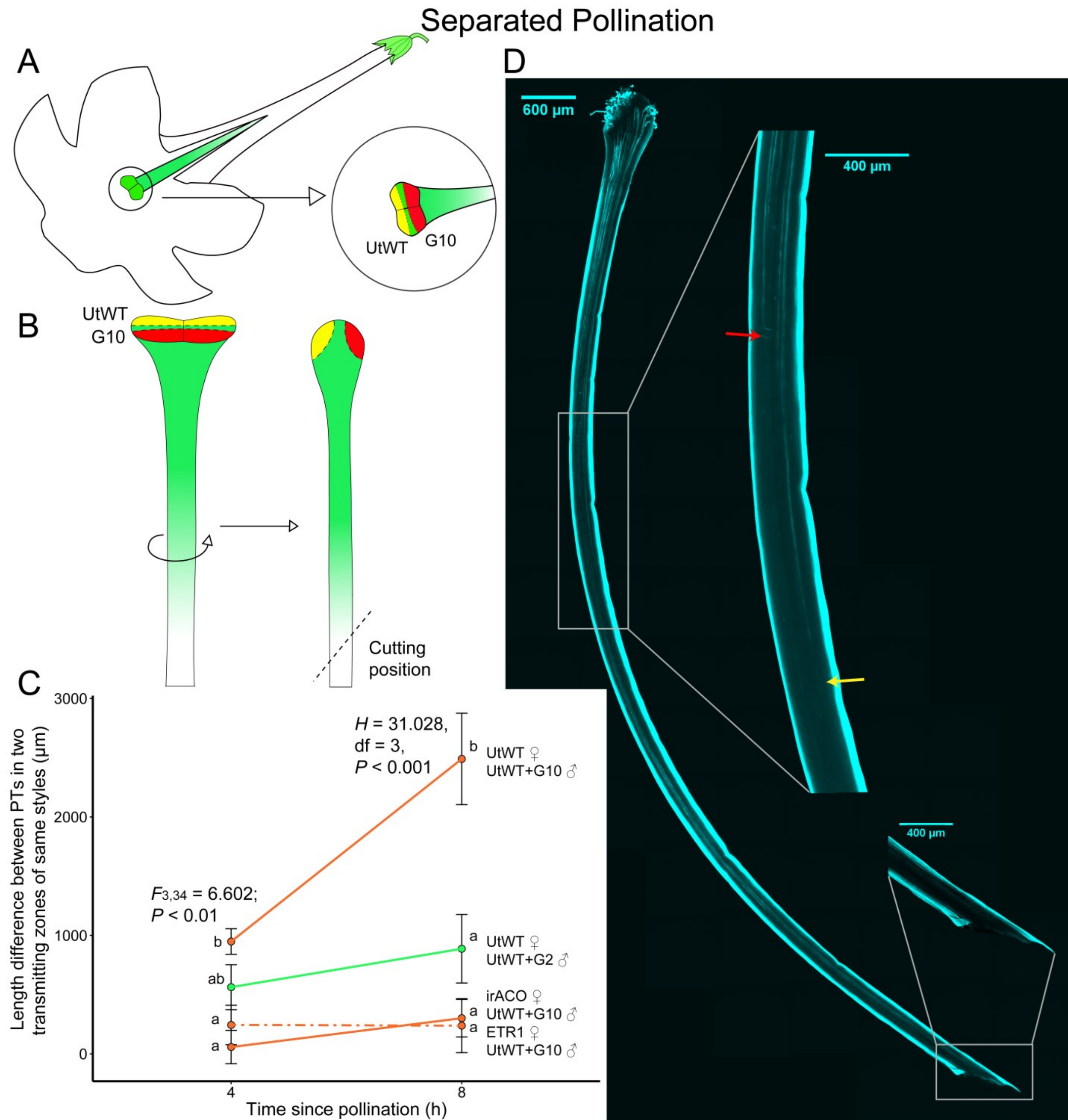


Figure 9. PT length difference between UtWT and G10 PTs in UtWT styles is the largest compared to the differences in non-selective maternal styles (irACO and ETR1) and the difference between UtWT and G2 PTs in UtWT styles 8 h after pollination. The length G10 and G2 PTs were constantly shorter than self pollen (UtWT) in UtWT styles at both time points. The length of G10 PT was shorter, equal or longer than the length of UtWT PT in

irACO and ETR1 styles at both time points. A) The cut on corolla for emasculation marked the zone of stigma pollinated with G10 (or G2) pollen. B) During sampling, the bottom of the style was cut at an angle of 45° to the vertical axis of style, and thus the pollination zone of stigma pollinated with G10 (or G2) pollen aligned with the obtuse angle formed at the style, while the other zone pollinated with UtWT pollen aligned with the acute angle at the bottom of the style. C) Statistical analyses of the length difference between two genotypes of PTs revealed that, at 4 h, the difference between UtWT and G10 PTs in UtWT styles is larger than the differences in non-selective maternal styles (irACO and ETR1). At 8 h, the difference between UtWT and G10 PTs in UtWT styles is larger than all the other three male-female combinations. D) An example microscopy picture illustrates the length difference between two genotypes of PTs. UtWT style was pollinated with UtWT and G10 pollen separately on the two transmitting zones. The sample was harvested 8 h after pollination. The red arrow indicates the ending position of G10 PT, while the yellow arrow shows the ending point of UtWT PT. The length between these two arrows was measured as length difference and used in the statistical analyses in Figure C.

Since the data set of the length difference of PTs at 8 h time point failed to pass the Shapiro-Wilk Normality test (W = 0.824, P < 0.001) but passed the Fligner-Killeen test of homogeneity of variances (χ^2 = 5.441, df = 3, P = 0.1422), we used Kruskal-Wallis rank sum test and Kruskal-Wallis multiple comparison instead of ANOVA and post hoc test. Kruskal-Wallis rank sum test and Kruskal-Wallis multiple comparison indicated that the length difference between UtWT and G10 PTs in UtWT styles was significantly larger than the length difference between UtWT and G2 PTs in UtWT styles and the length difference in non-selective styles (Fig. 9; H = 31.028, df = 3, P < 0.001), whereas, in non-selective styles, no statistical significance was found in the comparisons between the length difference of UtWT and G2 PTs in UtWT styles and the length difference of UtWT and G10 PTs. At 8 h, more samples showed unclear gaps between the two competitive PT bundles *in vivo*. UtWT styles pollinated with UtWT+G10 had the lowest proportion of samples with unclear gaps. However, the same genotype of styles pollinated with UtWT+G2 showed the highest proportion of samples with unclear gaps (Table S1).

As reported by Bhattacharya and Baldwin (2012), when self and non-self mixed pollination was performed on UtWT flowers, UtWT selects for self pollen. However, among non-self pollen, G2 was selected more by UtWT maternal plants compared with G10 pollen. The length of G10 and G2 PTs were constantly shorter than self pollen (UtWT) in UtWT styles. It indicated that the

differential PT growth rates of the two competitive PT donors were a factor involved in nonrandom seed siring. Moreover, Bhattacharya and Baldwin (2012) also demonstrated that ethylene-defective lines (irACO and ETR1) lost the ability of mate selection, and when UtWT+G10 mixed pollination was conducted on these two maternal lines, the two paternal genotypes sired, in average, equal amount of seeds. In irACO and ETR1 styles, the length difference between two pollen donors in the pollination combination UtWT+G10 was significantly smaller than the difference between these two donors in UtWT pistils. Furthermore, the longest G10 PT was shorter, equal or longer than the longest UtWT PT in irACO and ETR1 styles. These two findings confirmed that the differential PT growth rates of the two competitive PT donors were involved in nonrandom mating. Furthermore, the significantly larger length difference between UtWT and G10 PTs in UtWT styles observed from separated pollination compared with the length difference between UtWT and G2 PTs in UtWT styles at 8 h time point corresponded to fewer seeds sired by G10 in the seed set compared with G2. This suggested the correlation between PT growth rates and seed siring.

4.1.5 *O*-acyl sugars were found as potential mate selection-related metabolites

In total, 2105 features were obtained from the analysis of stylar tissues with UHPLC-ESI/HR-Q-TOF-MS. A t-test between UtWT styles (with stigmas) and irACO styles (with stigmas) sampled 8 h after being pollinated with UtWT+G10 revealed 83 features that significantly changed more than 2-fold. Furthermore, the comparison of UtWT styles pollinated with UtWT+G10 or with UtWT+G2 pollen mixtures indicated 34 features that were differentially regulated more than 2-fold. Twenty-six of these differentially regulated features were shared by these two comparisons (Table 1). These 26 features belong to 5 molecules and all of them were up-regulated with the same pattern in UtWT styles pollinated with UtWT+G10 pollen (Fig. 10). According to Arrendale *et al.* (1990), Weinhold and Baldwin (2011) and Luu *et al.* (2017), fragment of *m/z* 205, corresponding to an acetylated fructose moiety, and a neutral loss of 116 amu, corresponding to a C_6 aliphatic acid, are characteristics of a type (Class 3 and Class 4) of *O*-acyl sugars, while neutral losses of 116 amu and 202 amu, corresponding to a non-acetylated fructose moiety, are characteristics of another type (Class 2) of *O*-acyl sugars (Supplementary

Fig. S4). By checking the mass spectra, it was concluded that these 5 molecules are 5 different *O*-acyl sugars. Luu *et al.* (2017) described 15 different *O*-acyl sugars from trichomes of leaves. Four of the 5 *O*-acyl sugars discovered in this research are the same with the ones from trichomes, whereas one of these 5 *O*-acyl sugars has a different *m/z* value from any of the ones found in trichomes, with a smallest 0.04 mass difference (Table 1).

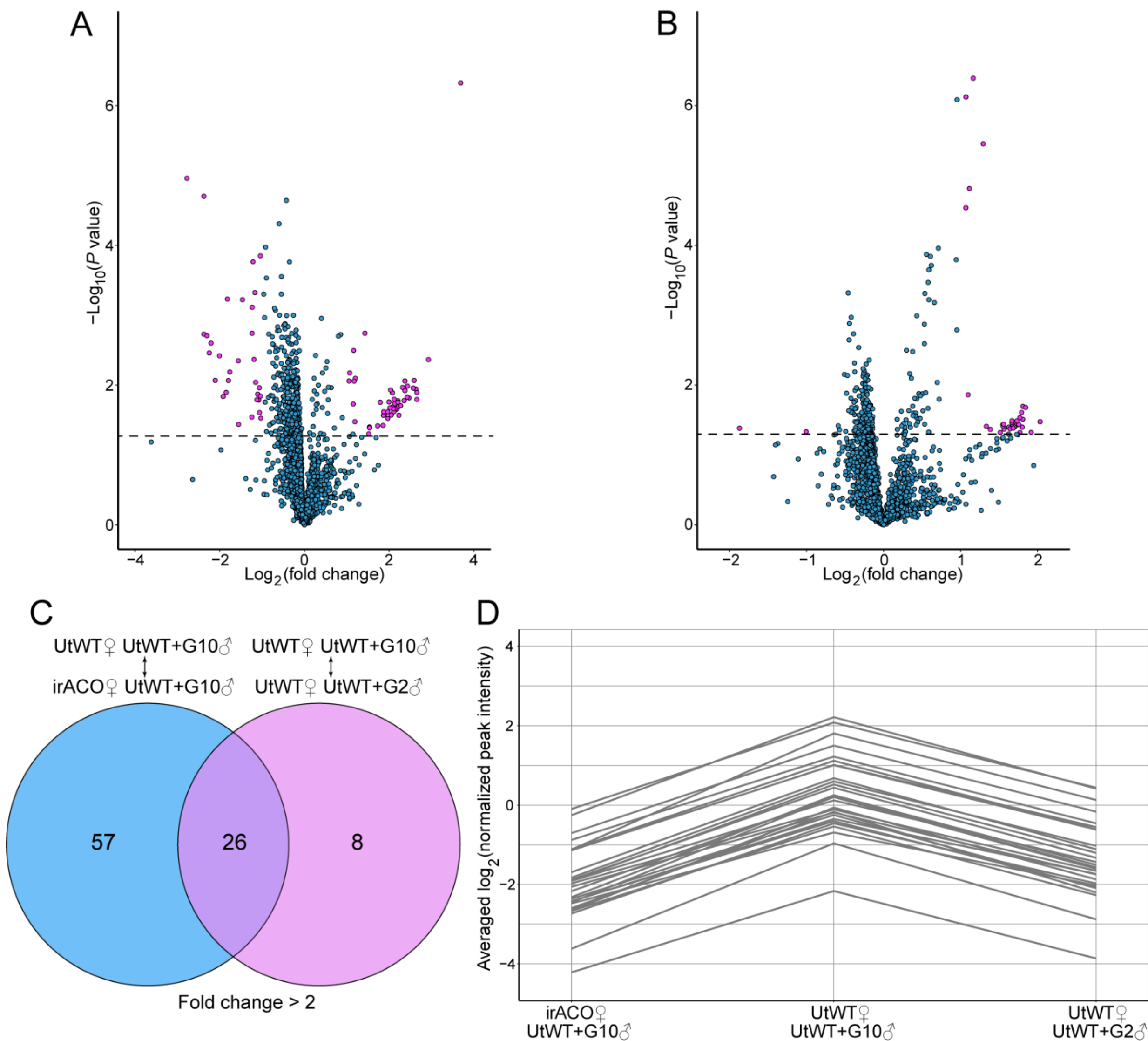


Figure 10. Five *O*-acyl sugars were indicated as potential mate selection-related secondary metabolites. UtWT styles (with stigmas) pollinated with UtWT+G10 or UtWT+G2, and irACO styles (with stigmas) pollinated with UtWT+G10 were sampled 8 h post-pollination and were extracted with 80% MeOH. Metabolome profiles were obtained by using Ultrahigh-performance liquid chromatography-electrospray ionization/high resolution

quadrupole time-of-flight mass spectrometry (UHPLC-ESI/HR-Q-TOF-MS). A) Volcano plot obtained from t-test between UtWT styles and irACO styles both pollinated with UtWT+G10 pollen summarizes both the fold-change (> 2) and the significance criteria (< 0.05). The pink dots refer to the features which passed the criteria indicated with the dashed line. B) Volcano plot generated from t-test between UtWT styles pollinated with UtWT+G10 and UtWT+G2 pollen indicates the features (pink dots) which significantly (< 0.05) differed more than 2-fold between groups. C) Venn diagram indicates the changed features shared by the two comparisons mentioned in A) and B). D) The change patterns of the 26 common features showed in the Venn diagram. All the features from five metabolites show the same expression pattern. The statistical results of t-test of the 26 features (Table 1), the mass spectra of the 5 *O*-acyl sugars (Supplementary Fig. S4) and the bar plots of the 5 parent ions (Supplementary Fig. S5) can be consulted in supplementary data.

To investigate the potential source of the *O*-acyl sugars, the metabolites of *in vitro* cultured PTs and unpollinated styles (with stigmas) were analyzed to dissect the PTs from the style tissue. The metabolomic profiles of the *in vitro* cultured PTs suggested that these 5 *O*-acyl sugars were present in PTs with trace amount. Each sample collected for PT metabolomic profile analyses contained the complete pollen from 10 anthers, but the pollen loaded on the stigma by hand pollination was dramatically less than pollen from 10 anthers. As reported in Bhattacharya and Baldwin (2012), each anther of *N. attenuata* contains around 4000 pollen grains. In this study, the maximum pollen amount loaded on a half of a stigma during hand pollination was 1218 among the checked samples (Table S3). Thus, the pollen loaded on one stigma by hand pollination should be no more than 3000. Therefore, the *O*-acyl sugars detected in pollinated styles were unlikely to be from pollen, but these compounds can be induced in PTs when the stylar tissue is present. Six unpollinated UtWT styles (with stigmas) were successfully measured separately. The metabolomic profiles of these six styles showed that the 5 *O*-acyl sugars were much more abundant than *in vitro* PT samples. In sum, the 5 *O*-acyl sugars are possibly present in styles and might be involved in mate selection. The functions of these *O*-acyl sugars in nonrandom mating should be tested in further studies.

Table 1. Statistical results of t-test of the 26 features potentially involved in mate selection and their presence in PTs and non-pollinated styles.

m/z	rt (min)	T-test between UtWT♀ UtWT+G10♂ and irACO♀ UtWT+G10♂				T-test between UtWT♀ UtWT+G10♂ and UtWT♀ UtWT+G2♂				Annotation	Presence in PTs	Presence in NP styles
		UtWT♀ UtWT+G10♂ $\log_2^{mean}$ (A)	irACO♀ UtWT+G10♂ $\log_2^{mean}$ (B)	$\text{Log}_2^{\text{fold change}}$ (A - B)	Adjusted *P*-value	UtWT♀ UtWT+G10♂ $\log_2^{mean}$ (A)	UtWT♀ UtWT+G2♂ $\log_2^{mean}$ (B)	$\text{Log}_2^{\text{fold change}}$ (A - B)	Adjusted *P*-value			
313.165	29.6	0.520	-1.911	2.431	0.010	0.520	-1.202	1.722	0.037	Class 2 *O*-acyl sugars, fragment	Yes[a]	Yes
631.296	29.6	-0.062	-2.650	2.588	0.009	-0.062	-1.871	1.809	0.031	Class 2 *O*-acyl sugars, [M+Na]+		
327.181	32	1.806	-1.121	2.927	0.004	1.806	0.130	1.677	0.037	Class 2 *O*-acyl sugars, fragment	Yes[a]	Yes
443.266	32	0.206	-2.449	2.655	0.013	0.206	-1.565	1.771	0.048	Class 2 *O*-acyl sugars, fragment		
645.312	32	0.250	-2.320	2.570	0.011	0.250	-1.505	1.754	0.037	Class 2 *O*-acyl sugars, [M+Na]+		
97.028	33.1	0.598	-1.826	2.425	0.015	0.598	-1.027	1.625	0.040	Class 3 *O*-acyl sugars, fragment	Yes[a]	Yes
99.08	33.1	-0.689	-2.479	1.789	0.018	-0.689	-2.024	1.335	0.039	Class 3 *O*-acyl sugars, fragment		
139.039	33.1	0.681	-1.690	2.371	0.012	0.681	-1.112	1.793	0.025	Class 3 *O*-acyl sugars, fragment		
205.071	33.1	0.440	-1.878	2.318	0.011	0.440	-1.329	1.769	0.029	Class 3 *O*-acyl sugars, fragment		
313.165	33.1	0.203	-2.167	2.371	0.009	0.203	-1.606	1.809	0.020	Class 3 *O*-acyl sugars, fragment		
345.139	33.1	-0.349	-2.423	2.075	0.013	-0.349	-1.735	1.386	0.043	Class 3 *O*-acyl sugars, fragment		
429.25	33.1	-0.170	-2.596	2.426	0.015	-0.170	-2.202	2.032	0.033	Class 3 *O*-acyl sugars, fragment		

668.351	33.1	-0.963	-3.618	2.655	0.016	-0.963	-2.878	1.915	0.047	Class 3 *O*-acyl sugars, fragment		
673.307	33.1	2.216	-0.254	2.470	0.015	2.216	0.415	1.801	0.040	Class 3 *O*-acyl sugars, [M+Na]+		
99.08	33.5	1.014	-1.140	2.153	0.022	1.014	-0.552	1.566	0.043	Class 2 *O*-acyl sugars, fragment	Yes[a]	Yes
341.197	33.5	2.082	-0.097	2.179	0.022	2.082	0.439	1.642	0.042	Class 2 *O*-acyl sugars, fragment		
139.039	34.3	0.120	-1.913	2.033	0.012	0.120	-1.431	1.552	0.039	Class 3 *O*-acyl sugars, fragment	Yes[a]	Yes
205.071	34.3	-0.110	-1.973	1.864	0.027	-0.110	-1.658	1.548	0.037	Class 3 *O*-acyl sugars, fragment		
327.181	34.3	-0.187	-2.057	1.870	0.024	-0.187	-1.745	1.558	0.036	Class 3 *O*-acyl sugars, fragment		
443.265	34.3	-0.537	-2.665	2.128	0.019	-0.537	-2.269	1.732	0.032	Class 3 *O*-acyl sugars, fragment		
687.323	34.3	1.499	-0.705	2.205	0.018	1.499	-0.165	1.665	0.034	Class 3 *O*-acyl sugars, [M+Na]+		
97.028	35.6	-0.403	-2.421	2.018	0.021	-0.403	-2.071	1.668	0.032	Class unknown *O*-acyl sugars, fragment	Yes[a]	Yes
139.039	35.6	-0.247	-2.364	2.117	0.016	-0.247	-2.093	1.845	0.021	Class unknown *O*-acyl sugars, fragment		
205.071	35.6	-0.459	-2.731	2.272	0.020	-0.459	-1.974	1.516	0.048	Class unknown *O*-acyl sugars, fragment		
696.383	35.6	-2.164	-4.213	2.049	0.027	-2.164	-3.863	1.699	0.040	Class unknown *O*-acyl sugars, fragment		
701.339	35.6	1.223	-0.876	2.099	0.022	1.223	-0.460	1.684	0.041	Class unknown *O*-acyl sugars, [M+Na]+		

[a] Features were present but with low abundance.

4.2 Paternal effects on seed metabolomes and differential pathogen resistance

4.2.1 Metabolomic profiles of the hybrid seeds showed clear differences

In total, 305 mass features were extracted from non-targeted metabolomics analyses after data filtering. Principal component analysis (PCA) indicates differences in metabolomic profiles of seeds with different paternities (Fig. 11). For seeds with Utah background (hybrid seeds obtained from UtWT maternal plants and paternal genotypes collected from Utah), clear separation along PC2 was detected among seeds sired by G2, G4 and the rest of the genotypes. Genotype G2, favored non-self pollen by UtWT, described above (Section 4.1) imparted its seeds with distinguishing metabolomic profiles compared with the seeds sired by G10, the non-favored non-self pollen donor described in Section 4.1. As to the seeds with Arizona background (hybrid seeds collected from AzWT maternal plants and Arizona paternal genotypes), seeds with different paternities were separated into distinct clusters along PC2. It is unclear if AzWT maternal plants select differentially on the 15 paternal genotypes as favored mate. *N. attenuata* UtWT plants, with obvious nonrandom mating pattern, might intend to equip their offspring seeds, with a certain metabolomic profile to adapt the challenges in the seed banks of their native habitats. However, solid conclusions cannot be drawn without testing the mate selection pattern on the 11 paternal genotypes and the abilities of their seeds to survive the stresses in the native habitats.

Seeds in seed banks may encounter many stresses, and fungal pathogen attack is one of them. In this research, I tried to answer the question whether the distinct constitutive metabolomic profiles of the seeds with different paternities confer the seeds with different pathogen resistance. Based on the separation of the seed metabolomic profiles, 4 genotypes of hybrid seeds with Arizona background (AA12, AA19, AA28, AA29) which represent the range of variation in PC2 (Fig. 11) were picked out for further pathogen resistance bioassays.

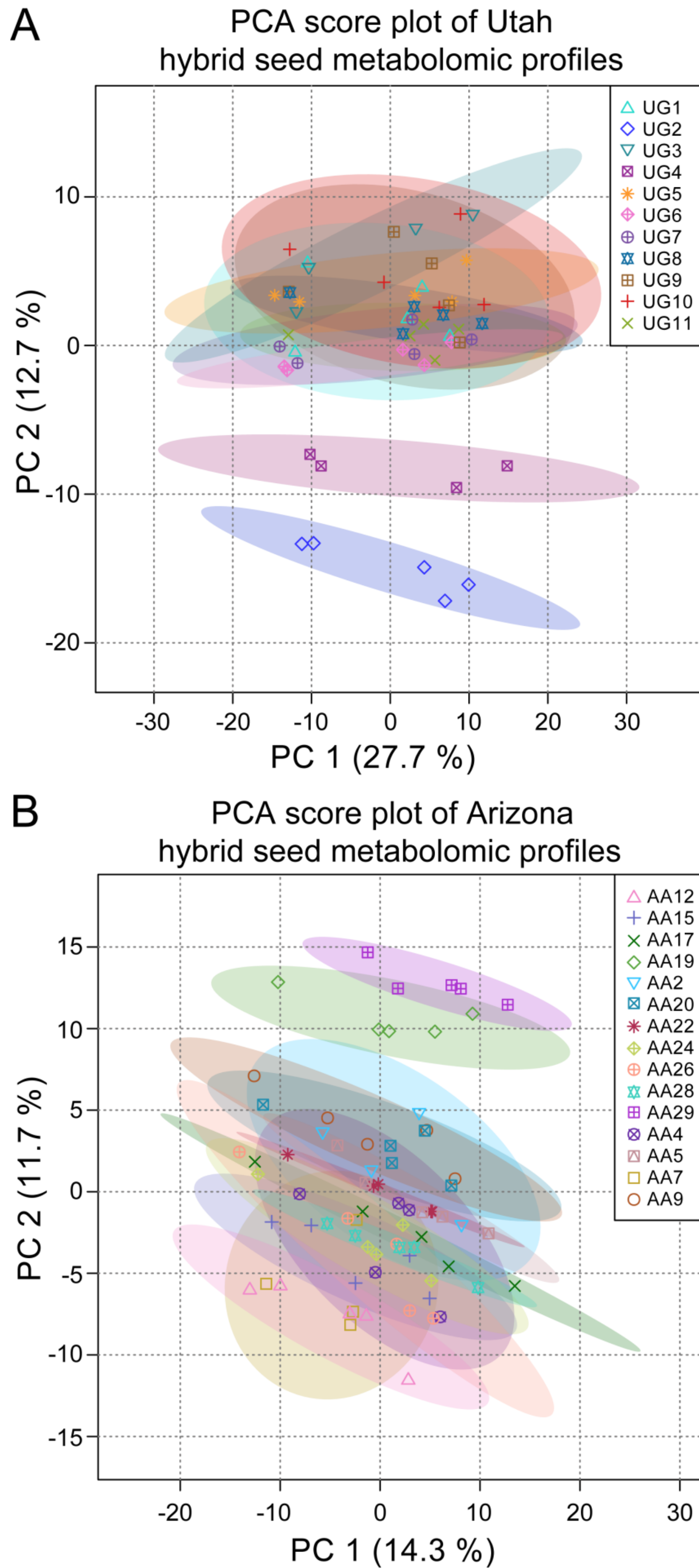
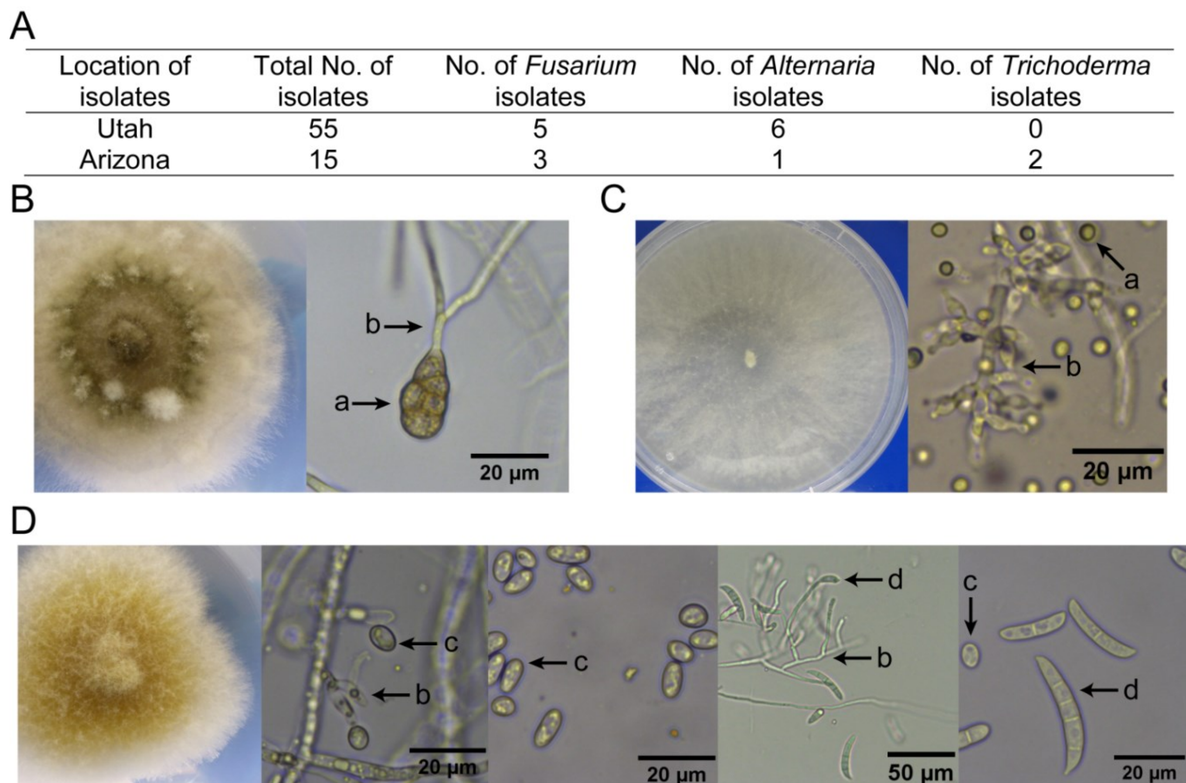


Figure 11. Principal component analysis (PCA) plots of metabolomic profiles of *N. attenuata* hybrid seeds. The seeds used in this analysis were obtained from flowers hand pollinated with different pollen donors and subsequently manually collected in the greenhouse. The first two components (PCs) explaining 40% and 26% of the variation were selected for the plots. Ellipses illustrate the 95% confidence ellipses. Each data point refers to a replicate used for analyses. A) PCA plot of metabolomic profiles of hybrid seeds with Utah background. UG1 to UG11 refers to seeds with UtWT maternity and G1 to G11 paternity, respectively. B) PCA plot of metabolomic profiles of hybrid seeds with Arizona background. AA12 refers to seeds with AzWT maternity and A12 paternity.

4.2.2 Selection of pathogens and different mortalities of different seeds

In total, 55 fungal isolates were obtained from the seeds buried in Utah, while 15 isolates were gained from the seeds buried in Arizona. Partial sequencing of internal transcribed spacer (ITS) sequences resolved some isolates at species level whereas some at genus level. Among the isolates, 5 from Utah and 3 from Arizona were identified as *Fusarium*. The 3 isolates from Arizona were the same species, *F. acuminatum*. Six of the isolates from Utah were classified as *Alternaria* (*A. burnsii or A. tenuissima*). From Utah, no *Trichoderma* was isolated, but in Arizona, two isolates (*T. hamatum and T. harzianum*) were identified as *Trichoderma* (Fig. 12A). *F. acuminatum*, *Alternaia* sp. and *T. harzianum* were chosen for further bioassays.



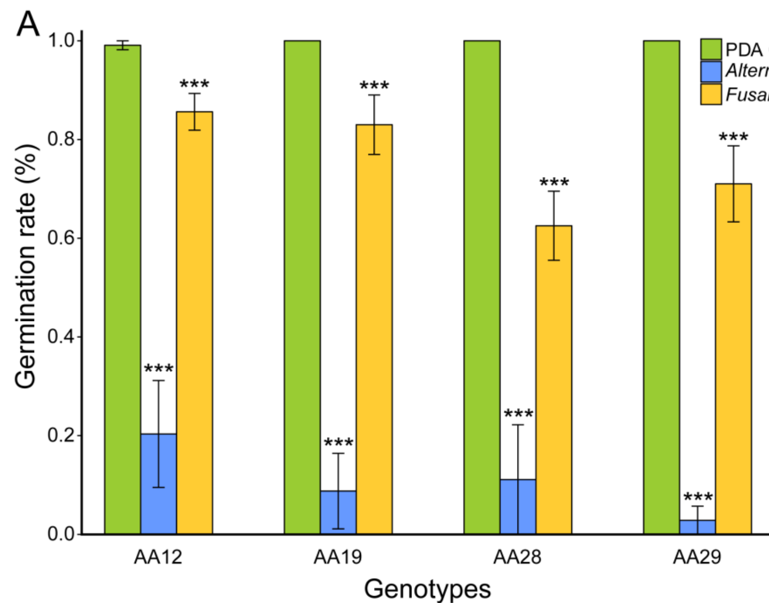
Location of isolates	Total No. of isolates	No. of *Fusarium* isolates	No. of *Alternaria* isolates	No. of *Trichoderma* isolates
Utah	55	5	6	0
Arizona	15	3	1	2

Figure 12. Morphologies of the 3 selected fungal isolates. Colony pictures were taken from the 7 days-old fungal cultures growing on PDA medium. A) The statistical descriptive information of the isolates obtained from the two locations. Fungal identification was performed molecularly by sequencing the internal transcribed spacer (ITS) regions. B) *Alternaria* sp. C) *Trichoderma harzianum*. D) *Fusarium acuminatum*. a, Conidia. b, Conidiophore. c, Microconidia. d, Macroconidia. Conidia, microconidia and conidiophores were obtained from 10 – 14-days-old fungal colonies cultured on PDA medium. The macroconidia were obtained from infected *N. attenuata* seeds germinated on GB5 medium for 10 days.

Before bioassay experiments, to confirm the accuracy of the ITS identification on the chosen isolates, fungal morphologies were observed (Fig. 12) under a light microscope. Conidia of the isolate identified as *Alternaria* sp. were ovoid with a short conical beak at the tip, which are the shapes characteristic of this genus (Schuck *et al.*, 2014). Conidia of the isolate designated as *T. harzianum* by ITS sequencing were yellowish green, smooth and subglobose. Its conidiophores showed regular dendroid branching patterns with short side-branches and short, swollen phialides. Both morphologies of conidia and conidiophores indicated that this isolate belongs to *Trichoderma* section *Pachybasium*. According to Bissett (1991), *T. harzianum* was classified into this section. The isolate classified as *F. acuminatum* by ITS sequencing showed abundant ellipsoid microconidia when it was growing on PDA medium, but no macroconidia were observed. The typical *Fusarium* banana-shaped macroconidia were observed from the infected seeds placed on Gamborg's B5 (GB5) medium. In sum, all the morphological characteristics of the 3 isolates are consistent with the ITS identification.

Alternaria sp. and *F. acuminatum* were used to infect *N. attenuata* seeds with distinct metabolomic profiles. Seeds after treatments were incubated in liquid smoke supplemented with GA_3 (1 mM) overnight. After the strong germination inducement, all alive seeds should germinate. Hence, the germination rate was used to indicate seed viability. Fisher's exact test indicated that all the genotypes of seeds infected with *Alternaria* sp. or *F. acuminatum* had significantly reduced viabilities ($P_{max} < 0.001$, Fig. 13A). However, more seeds germinated in the group of seeds inoculated with *F. acuminatum* than the seeds inoculated with *Alternaria* sp. (Fig. 13 and Fig. S6). Therefore, *Alternaria* sp. was defined as a virulent pathogen for *N. attenuata* seeds, whereas *F. acuminatum* was considered as a weak pathogen. When seeds were inoculated with *Alternaria* sp., no significant difference of survivorship of the seeds was detected among different seed genotypes (pairwise Quasibinomial GLM, df = 9, $t_{min} = -1.527$, $P_{min} = 0.165$). On the contrary, when seeds were infected with *F. acuminatum*, the significant difference of seed germination rate was detected between seed genotype AA12 and AA28 (pairwise Quasibinomial GLM, df = 9, $t_{AA12\&AA28} = -3.156$, $P_{AA12\&AA28} < 0.05$) but not between other pairwise comparisons (Fig. 13B). Albeit different genotypes of seeds showed no

differential resistance to the virulent pathogen *Alternaria* sp., they presented distinct resistance to the weak pathogen *F. acuminatum*. Hence, paternity could influence the susceptibility of the seeds to a weak fungal pathogen, *F. acuminatum*, but not to the virulent fungal pathogen, *Alternaria* sp..



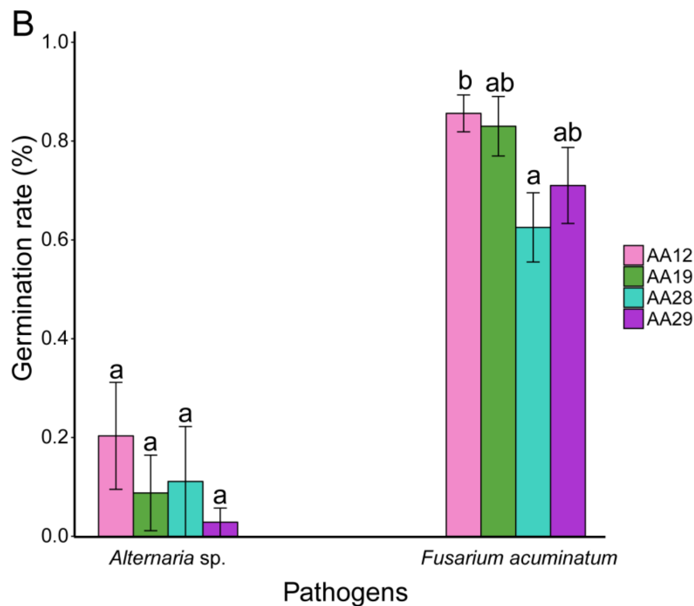


Figure 13. Fungal pathogenicity and pathogen resistance of different genotypes of seeds. Four different hybrid seeds (AA12, AA19, AA28, AA29) with distinct metabolomics profiles were selected for fungal pathogen bioassays. Imbibed seeds were incubated with fungus or PDA control medium in petri dishes for 7 days. For each combination of pathogen and seed genotype, 5 independent plates (replicates) were used. In each replicate, around 20 seeds were used. After incubation, seeds were germinated to record viabilities. A) The germination rates of all the genotypes of seeds infected with *Alternaria* sp. or *F. acuminatum* were significantly reduced compare with the control seeds (Fisher's exact test, P_{max} < 0.001). B) Different genotypes of seeds displayed different resistance to *Fusarium acuminatum* but not to *Alternaria* sp. (binomial GLM, standard errors were corrected using quasi-GLM models, $t_{AA12\&AA28}$ = -3.156, $P_{AA12\&AA28}$ < 0.05).

4.2.3 Constitutive metabolites potentially contributing to differential pathogen resistance of seeds

Since the strongest difference in seed viabilities was observed between AA12 and AA28 when the seeds were treated with *F. acuminatum*, the features or metabolites separating the metabolomic profiles of these two seed genotypes were the features that are worth a closer look. PC4 shown in Figure 14A is the PC that meets this criterion. Based on PC4, 37 features, with the absolute values of PCA loadings greater than 0.09 (Fig. 14B), were selected for further analyses. These features were considered as the ones contributing the most to the separation of the seeds along PC4. Therefore, these features might be the seed metabolites related to the different resistance to *F. acuminatum*.

For further visualizing the expression patterns of the 37 selected features in different genotypes of the hybrid seeds, hierarchical clustering was conducted to group the samples based on the expression levels of the 37 features, and to group the features with similar expression patterns across the samples. A heatmap together with two dendrograms were generated (Fig. 14C). The samples (replicates, in columns) were clearly grouped into 4 monophyletic clades which indicated that AA28 was prominently different from the other three genotypes of seeds. Features (metabolites) displayed in rows were clustered into 6 groups based on their expression patterns across the samples. According to the results displayed in Figure 13B, AA28 should present the biggest difference from AA12, but the smallest difference from AA29. Therefore, the features with expression levels indicating the largest difference between AA28 and AA12 but the smallest difference between AA28 and AA29 are considered as the features most likely relevant to differential pathogen resistance. Three groups of features (22 features) illustrated in Figure 14C (marked with red lines) met this criterion and are worth further investigations (Table 2 for more details). According to the *N. attenuata* online database, all these 22 features are unknown. Further studies to identify these features will help to reveal if they are involved in manipulating the abilities of the seeds to resist *F. acuminatum*.

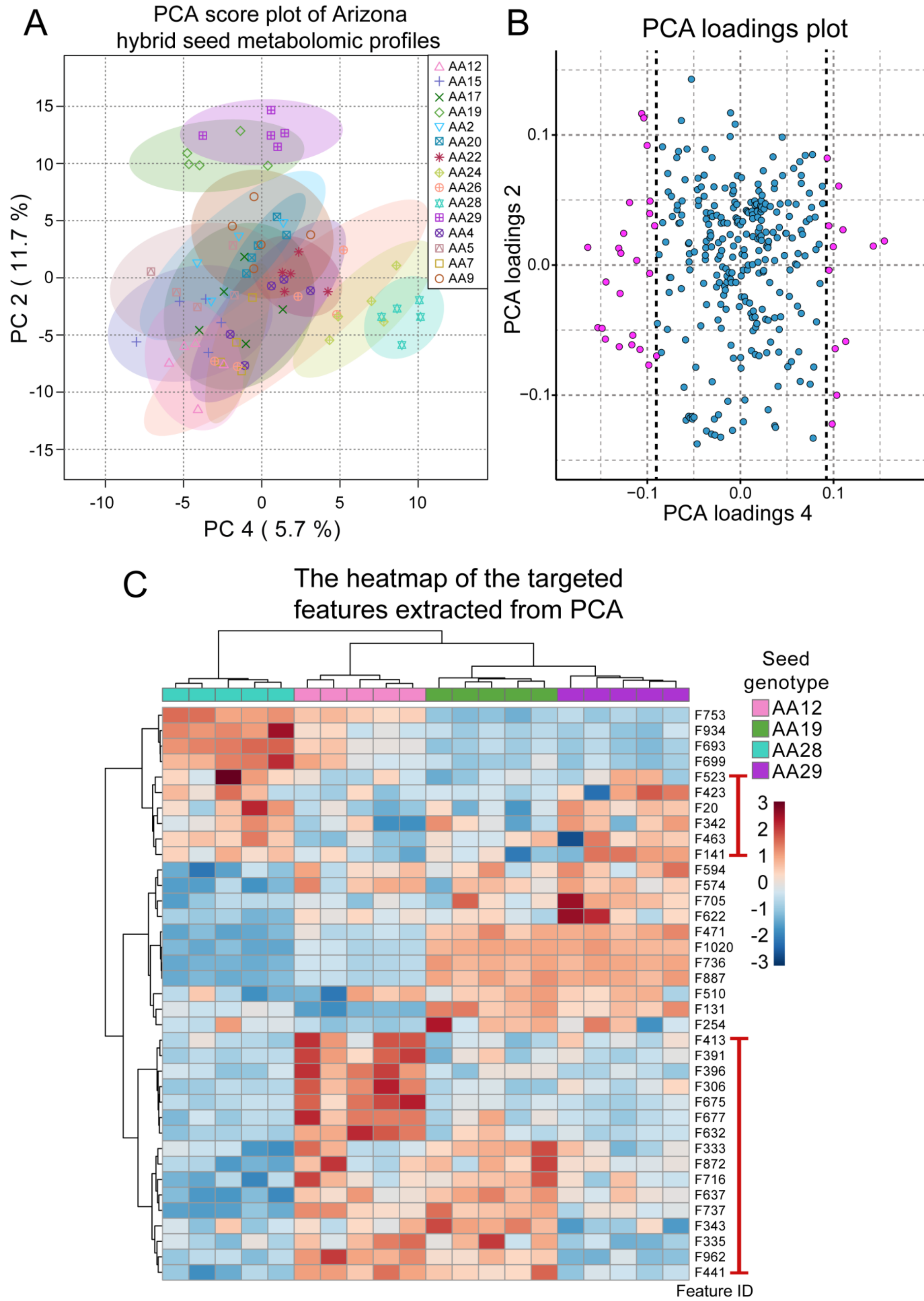


Figure 14. Features might potentially function as constitutive seed metabolites that confer different genotypes of seeds with distinct pathogen resistance (in Fig. 13B). Metabolites were extracted from imbibed seeds without

fungal infection. A) PCA plots of metabolomic profiles of *N. attenuata* hybrid seeds. Ellipses indicate the 95% confidence areas. Each data point on the figures refers to a replicate used for analyses. The biggest separation between AA12 and AA28 is shown along PC4. B) PCA loadings plot with loadings along PC4. The pink dots refer to the features with absolute values of the loadings along PC4 greater than 0.09. These features were selected to generate the heatmap. C) Heatmap and dendrograms constructed based on the features derived from PC4 of PCA. Hierarchical cluster analysis (Euclidean distance and Wad's linkage) generated 4 monophyletic clades of the samples (in columns) and 6 groups of features (metabolites, in rows). The intensities of reddish orange and blue represent an increase and decrease of the expression of a metabolite, respectively, relative to the median metabolite expression levels. The red lines on the right marked the features of interests and more details are shown in Table 2.

Table 2. The list of features of interests with *m/z* values and retention time that were selected by PCA and heatmap visualization in Figure 14.

Group	Feature ID	*m/z*	rt (min)	Annotation
Group I	F463	432.172	1.1	Unknown
	F523	458.186	1.1	Unknown
	F20	116.071	1.1	Unknown
	F423	411.141	1.3	Unknown
	F141	217.068	1.3	Unknown
	F342	355.246	7.9	Unknown
Group II	F396	383.131	2.8	Unknown
	F413	399.142	5.0	Unknown
	F306	331.154	6.3	Unknown
	F677	561.209	7.1	Unknown [M+Na]+
	F632	539.227	7.1	Unknown [M+H]+
	F391	381.132	7.3	Unknown
	F675	559.194	8.1	Unknown
Group III	F637	543.132	1.0	Unknown
	F333	353.121	2.5	Unknown
	F343	356.125	3.5	Unknown
	F335	353.137	6.2	Unknown
	F962	905.508	6.4	Unknown [M+H]+
	F872	741.442	6.4	Unknown
	F441	417.337	6.4	Unknown
	F716	579.388	6.4	Unknown
	F737	599.413	6.4	Unknown

4.2.4 *Trichoderma harzianum* inhibited the growth of the fungal pathogens

Dual culture assays revealed that *T. harzianum* isolated from Arizona could suppress the growth of both *F. acuminatum* and *Alternaria* sp. *in vitro* (Fig. 15). In the tests, only two independent replicates of each treatment of controls were conducted. Three independent replicates of the dual culture assay between *T. harzianum* and *Alternaria* sp., while two replicates of the assays between *T. harzianum* and *F. acuminatum* were performed. Due to the lack of replicates, statistical analyses were not performed to test the significance of the reduced colony growths of the pathogens inhibited by *T. harzianum*. However, as elucidated in Figure 15, at 3 days, *T. harzianum* was able to reduce the radial growth of *Alternaria* sp. (percent inhibition, 30.28%) more dramatically than it affected the radial growth of *F. acuminatum* (percent inhibition, 6.12%). A clear inhibition zone could be observed between the colonies of *T. harzianum* and *F. acuminatum* 3 days after co-culturing (Fig. 15A), but not between the colonies of *T. harzianum* and *Alternaria* sp. (Fig. 15B). Prominent mycoparasitism between *T. harzianum* and *Alternaria* sp., where the hyphae of *T. harzianum* coiled around the hyphae of *Alternaria* sp., could be observed under the stereomicroscope (Fig. 16B). Nevertheless, the mycoparasitism between *T. harzianum* and *F. acuminatum* seemed to be hindered probably by the extracellular secretion on *F. acuminatum* hyphae (Fig. 16A). Six days after culturing, the radial growth of *Alternaria* sp. was inhibited 57.24% by *T. harzianum*, whereas the radial growth of *F. acuminatum* was hindered similarly with 40.00% inhibition. The inhibition zone between the colonies of *T. harzianum* and *F. acuminatum* became smaller and more blurred (Fig. 15A). Since *T. harzianum* had started sporulating at this stage, the mycoparasitism could not be observed easily. Therefore, whether *T. harzianum* could establish mycoparasitism on *F. acuminatum* 6 days after culturing remained elusive. In sum, the tests suggested that *T. harzianum* could inhibit the growths of the two tested pathogens *in vitro*.

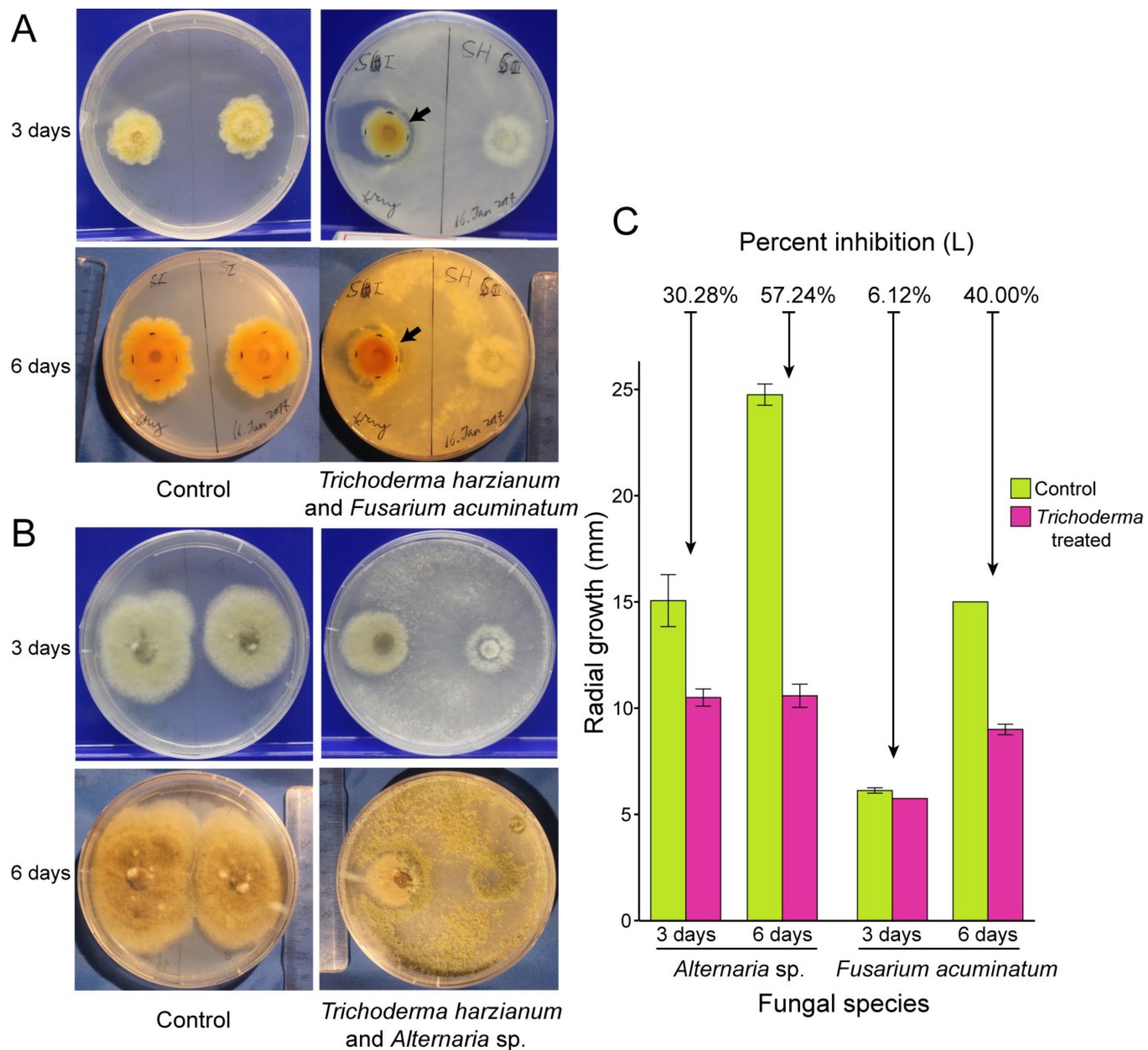


Figure 15. Dual culture assays revealed that *T. harzianum* could inhibit the growth of the fungal pathogens. A mycelial plug of *T. harzianum* (on the right side in the figure) and a same-sized mycelial plug of the pathogen (on the left side in the figure) were placed on the opposite two sides of a PDA plate. The control was a plate with two same-sized mycelial plugs of the same pathogen on the two halves of the plate. A) Representative pictures of the dual culture assay between *T. harzianum* and *F. acuminatum*. The black arrows indicate the inhibition zones between the colonies. B) Representative pictures of the dual culture assay between *T. harzianum* and *Alternaria* sp.. C) Bar plot of the inhibition of the radial growth of the pathogens. The growth of *Alternaria* sp. was hindered starting from day 3, the inhibition on *F. acuminatum* started at day 6. Because of lack of replicates (2 replicates for controls and two replicates for dual culture assays between *T. harzianum* and *F. acuminatum*), statistical analyses were not performed.

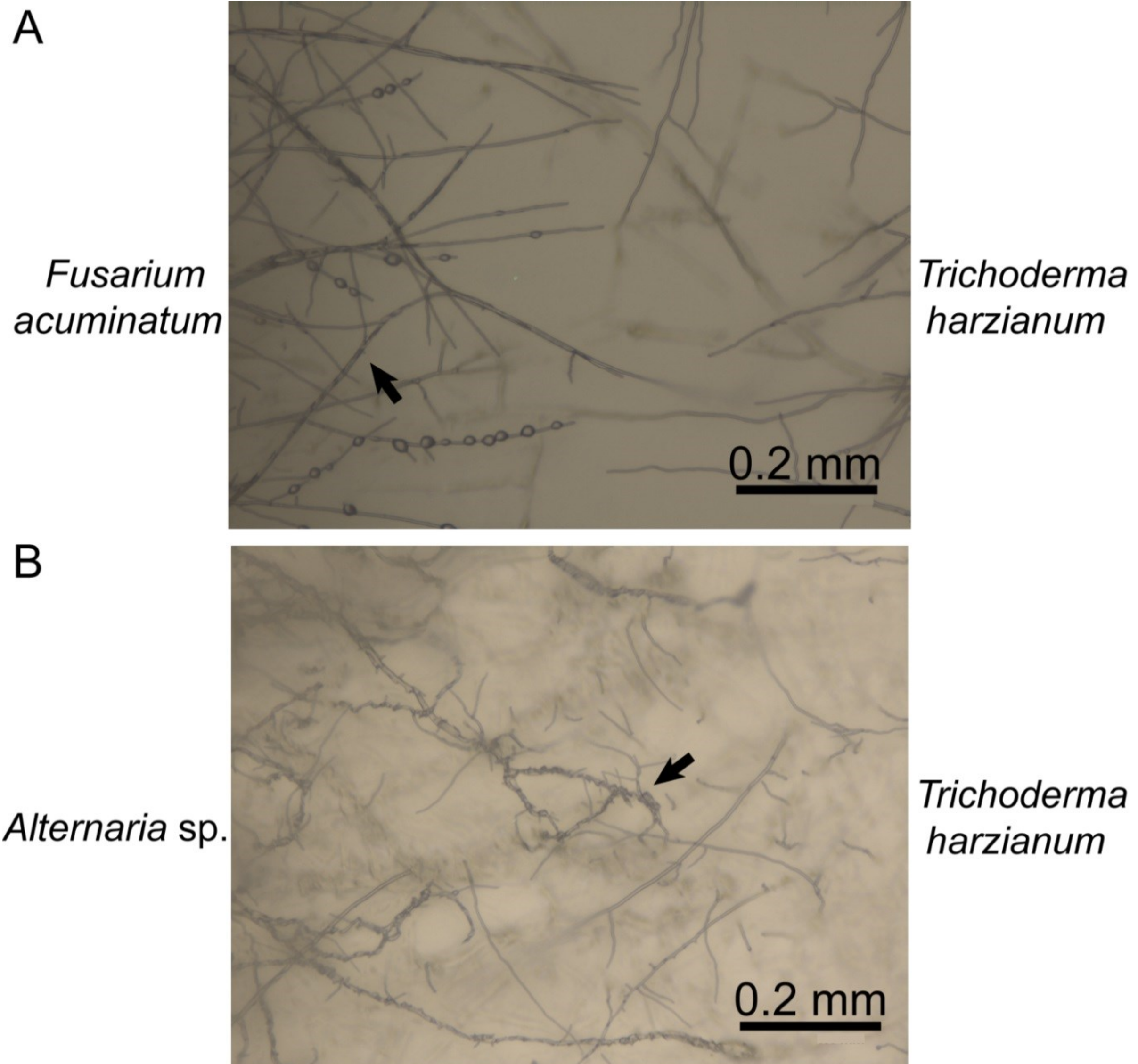


Figure 16. Mycoparasitism visualization between *T. harzianum* and seed pathogens. The interacting areas between *T. harzianum* and the pathogens 3 days after culturing were viewed under the stereomicroscope. Pathogen colonies were located on the left side of the field, while *T. harzianum* was on the right. Black arrows refer to the visible mycoparasitism. A) Mycoparasitism between *T. harzianum* and *F. acuminatum*. The formation of the mycoparasitism was hindered. B) Mycoparasitism between *T. harzianum* and *Alternaria* sp..

5. Discussion

5.1 *In vivo* pollen tube growth rate and nonrandom mating in *N. attenuata*

In single pollinations, pollen germination rates and PT growth rates showed no difference at any time point. At 1 h, self pollen had relatively lower germination rate on UtWT styles compared with cross pollen, but this difference is not significant. Equal PT growth rates in single pollinations are consistent with the result from Bhattacharya and Baldwin (2012) that different genotypes of pollen donors show equal fecundity in single pollinations. However, in each pollination, the PT growth rate indicated high variance. For example, at 8 h in UtWT flowers, the length of UtWT PT in single pollination varied from 6357.6 μm to 11441.46 μm, while G10 PT varied from 2414.1 μm to 12736.99 μm, and G2 PT varied from 1298.12 μm to 11147.32 μm. Kessler *et al.* (2012) discover that, in *N. attenuata*, nectar nicotine concentrations are highly variable, and no predictable spatial patterns in regard with ontogeny or floral meristem location are found. It suggests that every flower is different. Although no statistically significant difference was detected, it has still not been demonstrated that the maternal flowers equally allow the PTs from different donors to pass through when flowers are singly pollinated, because the variances within pollination treatments are huge. The difference may become significant when large sample size is included.

The large variance among samples within the same treatment suggests a drawback of predicting the PT performances *in vivo* in mixed pollination by using single pollination. On the other hand, the effect of pollen-pollen interaction is unable to be detected by only investigating single pollination (Aizen *et al.*, 1990; Cruzan, 1990; Marshall *et al.*, 1996; Varis *et al.*, 2010). Therefore, visualizing two competitive pollen donors growing in the same style is the optimal approach to address if PT growth rates of different donors are different *in vivo*.

As far as I am aware, this study is the first to visualize the differential PT growth rates of different natural pollen donors without genetic transformation within a syncarpous gyneocium with a single solid style. In UtWT pistils, the longest G10 PT was constantly shorter than the longest UtWT PT within the same style. However, the magnitude of the difference between G10

and UtWT PTs was variable. This variance was consistent with the variance of the seed set. In irACO and ETR1 styles (the lines lose nonrandom mating), the length difference between two pollen donors in the same pollination combination (UtWT+G10) was significantly reduced. Furthermore, the longest G10 PT was not constantly shorter than the longest UtWT PT in irACO and ETR1 styles. The findings suggest that the female choice might have great influence on PT growth rates, and ethylene signaling might influence the PT growth rates. On the other hand, the length difference between donors in different pollination combinations (UtWT+G2 and UtWT+G10) presented significant difference in the same type of maternal pistils (UtWT). It indicates that either the female might choose the male differently according to the male genotypes or the PTs from different donors might have different competitive abilities. Bhattacharya and Baldwin (2012) found that the favored mixed-pollination combination (UtWT+G2) and non-favored mixed-pollination combination (UtWT+G10) in UtWT flowers could be reversed in other natural maternal genotypes. This implicitly suggests that, possibly, the female choice is the only factor that affects nonrandom mating, and it might regulate the male-male competition. Since, in plants, male-male competition, if exists, mainly occurs in the female tissue (pistil), it is extremely challenging to separate these two processes. Further studies are required to unravel the puzzle.

In separated pollination, I found that not every style checked showed clear separation of two bundles of PTs (Supplementary Fig. S3 and Table S1). In some styles, the PTs of two pollen donors mixed together at 8 h, and they showed unclear gap between two types of PT bundles. At 4 h, only one sample showed unclear gap between two types of PT bundles. At 8 h, the lowest proportion of samples with unclear gaps was found in UtWT styles pollinated with UtWT+G10, while the highest proportion of samples with unclear gaps was discovered in UtWT styles pollinated with UtWT+G2. It is technically very difficult in distinguishing pollen identities of different pollen donors in mixed pollinations. The pollination method and the sample mounting method developed in this research are useful to study the correlation between PT growth rate and seed siring, but it has its limits. At early time point, the identities of PTs were

distinguished effectively. At late time point, the ability of the PT-identity distinction was not efficient in UtWT styles pollinated with UtWT+G2 pollen due to the entangling of PTs.

The unclear separation of two donors of PTs at later time point (8 h) might be caused by several reasons. Firstly, since the space within a style is limited, the gap between two PT bundles may be squeezed into a thin invisible film by excessive amount of PTs. As introduced before, G2 pollen is considered as favored non-self pollen and UtWT+G2 mixed pollination is considered as favored pollination combination (Bhattacharya and Baldwin, 2012). I hypothesize that the UtWT styles pollinated with favored UtWT+G2 pollination combination have more germinated pollen grains on the stigmas or lower level of PT attrition than the styles pollinated with UtWT+G10, the non-favored pollination combination. Consequently, higher pollen germination rates or lower levels of PT attrition in these samples may result in more samples with unclear gaps between two PT bundles from different donors.

Pollen-germination ability can be influenced by paternal genotype, maternal genotype and the presence of other pollen, and thus lead to nonrandom mating (Cruzan, 1986; Bertin and Sullivan, 1988; Marshall *et al.*, 1996; Nemeth and Smith-Huerta, 2002; Jolivet and Bernasconi, 2007; Swanson *et al.*, 2016). Therefore, perhaps, the presence of G2 pollen in the favored pollination combination (UtWT+G2) increased pollen germination rate on UtWT stigma. Consequently, excessive amount of PTs in the style at 8 h increased the proportion of samples with unclear gaps in styles pollinated with UtWT+G2. However, to test this hypothesis is not feasible. I estimated the number of pollen grains on one transmitting zone of the stigma in separated pollination (Supplementary Table S3). Although most of the trials showed similar amount of pollen grains loaded on one half of the stigma, it is challenging to load equal amount of pollen on the stigma. Moreover, it is even more challenging to estimate the amount of germinated pollen separately on two halves of a stigma. Hence it was challenging to estimate and conclude whether the higher germination rate of pollen in favored pollination caused the higher proportion of samples with unclear gaps in favored pollination combination compared with non-favored pollination combination.

PT attrition is a phenomenon where the number of growing PTs decreases during growth through the style (Herrero and Dickinson, 1981; Cruzan, 1986; Snow, 1986; Cruzan, 1989, 1990; Scribailo and Barrett, 1991; Montalvo, 1992; Cruzan and Barrett, 1993; Plitmann, 1993; Winsor and Stephenson, 1995; Cruzan and Barrett, 1996). The physical limitation on the width of the stylar transmitting tissue or the limitations on the nutrient supplies to pollen restrains the number of PTs that can grow through the style (Marshall and Folsom, 1991; Stephenson *et al.*, 2003; Mazer *et al.*, 2016). As it was almost impossible to record the number of PTs in one half of a style at different distances away from the stigma, it was difficult to conclude whether the level of PT attrition was different between favored pollination combination (UtWT+G2) and non-favored pollination (UtWT+G10). Therefore, further studies are required to conclude that if the samples with unclear gaps were caused by excessively large amount of PTs growing in the styles, and further to demonstrate that whether more vigorous PT growth or pollen germination rate was associated with the weaker level of mate selection.

Secondly, more severe programmed cell death (PCD) of the UtWT transmitting tissue elicited by favored pollen combination (UtWT+G2) may be a reason for the highest proportion of samples with an unclear gap between PTs. In *Arabidopsis thaliana*, PCD of the transmitting tract can be accelerated by pollination (Crawford *et al.*, 2007). In *N. tabacum*, it has been suggested that incompatible pollen does not promote PCD, and the growth of the PT itself may elicit the breakdown of the stylar transmitting tissue (Wang *et al.*, 1996). Therefore, in this study, the favored non-self pollen, G2, on UtWT styles might have caused more severe PCD in the stylar transmitting tissue, and thus more samples with unclear gaps between PT bundles were observed at 8 h. Wang *et al.* (1996) also proposed that part of the PCD process requires ethylene signaling, since blocking ethylene receptors leads to an incomplete cell death process in the transmitting tissue. Bhattacharya and Baldwin (2012) demonstrated that a favored pollen combination (UtWT+G2) on UtWT stigmas induces a larger post-pollination ethylene burst (PPEB) than non-favored pollen combination (UtWT+G10) does. Therefore, the high ethylene emission of the UtWT styles pollinated with UtWT+G2 might have triggered more severe PCD in

the transmitting tissue at later time point, and hence caused PTs from two donors tangled together, and thus more samples with unclear gaps. More studies should be performed to investigate if PCD is linked with nonrandom mating in *N. attenuata*.

At last, since I cannot exclude PT-PT interaction conclusively in this study, the clear gap between PT bundles of two competitive donors might also be caused by male-male interference competition in *N. attenuata*. There are two types of male-male competition among pollen. One is known as exploitation competition, which refers to the races for access to ovules (e.g. the differential growth rates). The other one is called interference competition, and it refers to the interference with the germination and growth of pollen from other pollen donors (Marshall *et al.*, 1996). Because PTs grow in female tissue and are heavily regulated by the pistils, it is difficult to separate female choice and male-male competition in plants. However, it is known that pollen exudates are involved in pollen-pollen interference competition where pollen from one donor affects the germination and growth of pollen from other donors (Marshall *et al.*, 1996; Pasonen and Käpylä, 1998; Varis *et al.*, 2010). The highest proportion of samples with clear gaps in UtWT styles pollinated with UtWT+G10 at 8 h is likely due to the strongest interference competition between UtWT and G10 PTs. The lower proportion of samples with clear gaps in ethylene-defective lines (irACO and ETR1) pollinated with UtWT+G10 may indicate weaker PT-PT interaction between UtWT and G10 PTs compared with the interaction in UtWT styles. It is reasonable to infer that ethylene signaling might be involved in the PT-PT interference competition. However, more studies should be conducted to reveal if male-male interference competition is implicated in nonrandom mating in *N. attenuata*.

In sum, in this study, it was discovered that the differential PT growth rates between self pollen (UtWT) and non-self pollen (G10 or G2) were involved in nonrandom mating in *N. attenuata*. The novel pollination method and the sample mounting method developed in this research were useful to observe *in vivo* differential PT growth rates at early time point. At late time point, the observation on UtWT styles pollinated with non-favored pollination

combination (UtWT+G10) was still ideal. However, at time points later than 8 h, it is likely that the distinction between these two pollen donors in UtWT styles will become less feasible and less ideal because of the increase of samples with unclear gaps between two PT bundles of these two donors. At 8 h, UtWT styles pollinated with UtWT+G2 showed the highest proportion of samples with unclear gaps between the two pollen donors. Therefore, 8 h or later time points are not ideal time points to observe PT exploitation competition *in vivo*, if UtWT styles are pollinated with UtWT+G2 pollen. It is challenging to unravel what reason causes the unclear gaps between two PT bundles of different pollen donors in styles. Aside from PT growth rates, it is unclear that if other factors of pollen and style performance, such as pollen germination, PT attrition and transmitting tissue degradation, are involved in the process of nonrandom mating in *N. attenuata*. Further investigations are needed to figure out the more detailed mechanism of nonrandom mating in *N. attenuata*.

5.2 Secondary metabolites and nonrandom mating in *N. attenuata*

This research is also the first one that tried to use metabolomics approaches to unravel the secondary metabolites implicated in mate selection. For the first time, *O*-acyl sugars were indicated as potential secondary metabolites involved in mate selection in *N. attenuata*. *O*-acyl sugars are viscous liquids that consist of aliphatic acids of different chain lengths esterified to sucrose. Many studies proposed that they are effective defense compounds against herbivores (Rodriguez *et al.*, 1993; Chortyk *et al.*, 1996; McKenzie and Puterka, 2004; Weinhold and Baldwin, 2011), fungal pathogens (Luu *et al.*, 2017) and bacteria (Chortyk *et al.*, 1993). However, no studies were performed to demonstrate that *O*-acyl sugars are involved in plant reproduction, let alone plant sexual selection. I detected that the relative concentrations of 5 different *O*-acyl sugars were much higher in UtWT styles pollination with non-favored pollen combination (UtWT+G10) than favored one (UtWT+G2) and higher than irACO styles pollinated with the same pollen combination (UtWT+G10). Therefore, hypothetically, it is likely that *O*-acyl sugars are involved in nonrandom mating in *N. attenuata* and the relatively high concentrations of these *O*-acyl sugars might inhibit the growth of G10 PTs.

Luu *et al.* (2017) discovered that *O*-acyl sugars have anti-fungal activities in *N. attenuata*. Fungal hyphae and PTs share many similarities. Both of them show tip growth and heterotrophism. Kessler *et al.* (2010) proposed that fungal pathogen infection and PT reception share molecular components. Many floral fungal pathogens often invade plants through the nectaries (Sasu *et al.*, 2010) or through the pistils (Ngugi and Scherm, 2006) of flowers, and then produce infectious units that hide from plant immune responses by mimicking PTs invading the pistils (Ngugi and Scherm, 2004). As *O*-acyl sugars have anti-fungal activities, it is possible that *O*-acyl sugars can hinder the growth of PTs or inhibit pollen germination as well. However, further bioassays are required to reveal the effects of *O*-acyl sugars on PTs. *O*-acyl sugars found in this research are also likely linked with ethylene signaling, because irACO styles pollinated with UtWT+G10 have lower concentrations of *O*-acyl sugars than the concentrations in UtWT styles with the same pollination. As discussed above, the highest proportion of samples with clear gaps between PT bundles was found in UtWT styles pollinated with UtWT+G10. It is reasonable to infer that the low concentrations of *O*-acyl sugars might be associated with the high proportion of samples with unclear gaps. Further research is needed to investigate how *O*-acyl sugars are involved in nonrandom mating.

5.3 Paternal effects on seed metabolites and seed resistance to pathogens

In this study, the metabolomic profiles of the imbibed hybrid seeds clearly showed that different paternal genotypes diversify their offspring seed metabolomes. To my knowledge, this is the first study to show the influence of different paternal lines on the metabolomes of their offspring seeds. Using the AzWT maternal line, offspring seeds sired by 15 natural paternal lines collected from Arizona showed obvious metabolomic diversity (Fig. 11). Similarly, using the UtWT maternal line, hybrid seeds sired by 11 natural paternal lines collected from Utah displayed clear metabolomic diversity (Fig. 11). Seed metabolomes include important reserves for growth, such as starch, lipids and proteins. These are crucial for germination and support early seedling establishment and growth (Terskikh *et al.*, 2005) Some seed metabolites are important attractants and signaling molecules for establishing symbiotic associations, or function as antimicrobial metabolites involved in seed defenses (Wijaya *et al.*, 2000; Ndakidemi

and Dakora, 2003; Fuerst *et al.*, 2011). While seeds sired by G2 (the non-self paternal genotype favored by the UtWT maternal line) on UtWT maternal line have distinct metabolomic profile compared with the seeds sired by G10 (the non-self paternal genotype not favored by the UtWT maternal line), it is reasonable to ask if the mate selection in *N. attenuata* is adaptive, if offspring seeds with different metabolomic profiles sired by different paternal lines adapt differently in their natural habitat. Many studies have shown that paternal genotypes affect the size of seeds and seedling performance (Bookman, 1984; Mazer *et al.*, 1986; Andersson, 1990). It is possible that *N. attenuata* might leverage nonrandom mating to allow beneficial pollen donors to sire more seeds with traits providing better adaptation in the native seed banks. However, the data collected in this study are not sufficient to answer these questions. At first, it should be clear whether the AzWT maternal line has different compatibilities with the 15 Arizona paternal lines in mixed pollination. Similarly, nonrandom mating by the UtWT maternal line with the 11 Utah paternal lines should be investigated. If consistent and clear nonrandom mating patterns are discovered in these two maternal lines when applying mixed pollinations, the hybrid seeds with different paternities could be buried in a natural seed bank to test their survivorship after a growing and dormant season. If the successful paternal genotypes in mixed pollination also produce offspring seeds with longer longevities in the seed bank, the conclusion can be drawn that the nonrandom mating in *N. attenuata* plants is adaptive and can enhance the performance of their offspring seeds in seed bank.

Although I could not clearly demonstrate the linkage between nonrandom mating in *N. attenuata* and the adaptive traits of seeds with different paternities in field, I isolated culturable fungi from the seeds buried in the native soil where the native seed banks are carried. Seeds in the native seed bank often face many challenges from soil-borne microbes. Investigating seed-microbe interactions in the lab may provide guidance for further field studies. A species of *Alternaria* isolated from Arizona was identified as a virulent pathogen for *N. attenuata* seeds. The resistance of seeds against this virulent pathogen did not differ by paternity or associated metabolomic profile (Fig. 13). However, *Fusarium acuminatum*, a weak pathogen isolated from Arizona, caused different mortalities on the tested seeds. Together

these assays indicate that the different paternities and seed metabolites may equip the seeds with different resistances against *Fusarium acuminatum* but not against *Alternaria* sp. (Fig. 13). In this research, only 5 independent replicates were used for each bioassay. More replicates should be tested.

F. acuminatum is commonly known as a weak pathogen in some plant species (Fernandez *et al.*, 1985; Axelrood *et al.*, 1995), and it is also known as a secondary invader associated with root rot diseases (Warren and Kommedahl, 1973). The association of *F. acuminatum* with another more virulent pathogen usually leads to augmentation of the rot diseases compared with the infection of each pathogen alone (Fernandez *et al.*, 1985; Mao *et al.*, 1998). In native seed banks, *F. acuminatum* and *Alternaria* sp. used in this study may associate with other microbes into different consortia, and thus have different effects on *N. attenuata* seeds. In this study, the pathogenicities of many other fungi isolated from the same area were not investigated. Besides, this study only focused on culturable fungi. Influence of microbes on seed-bank survival is still poorly understood for *N. attenuata* seeds, and the assays in this study did not address the influence of bacteria or any unculturable microbes. Although this study cannot provide a detailed picture about how seeds of *N. attenuata* interact with the deleterious microbes, it brings a new perspective to understand the survival of seeds in native seed banks. For future studies, the interaction of the seeds with other microbes alone or the consortia of them can be investigated in the lab, and in the field under natural conditions.

In this study, 22 features of seed metabolites were selected by analyses as compounds that might be involved in differential fungal pathogen (*F. acuminatum*) resistances of the 4 genotypes of seeds tested (Fig. 14 and Table 2). The structures and the identities of the 22 features are unknown. Unraveling the identities of the 22 features may help to understand the functions of these seed metabolites. Some of these metabolites might function as anti-fungal compounds and might be involved in seed defense against *F. acuminatum* tested in this study. However, the analyses of this study did not cover the whole spectrum of metabolites from seeds. The MeOH based extraction buffer (50% MeOH) is not efficient at extracting highly

lipophilic seed metabolites. Moreover, the column and the mobile phase combination used in this research elutes the most polar metabolites all at once at the beginning of the chromatographic run making highly polar metabolites, such as amino acids, small peptides, monosaccharides and other small sugars, difficult to analyze. Further, this approach is not appropriate for the analysis of many biological macromolecules, such as proteins and polysaccharides. All these biological molecules may be involved in plant defenses against fungi. For example, some seed proteins, such as lectin, are involved in seed defense against fungal pathogens (Terras *et al.*, 1992; Freire *et al.*, 2002). Some fatty acids on leaf surface have anti-fungal activities, as well (Franich *et al.*, 1983; Kato *et al.*, 1983; Kato *et al.*, 1993; Namai *et al.*, 1993). Therefore, to fully understand the mechanisms of seed defense, several methods of extraction and analysis should be combined for further studies. Aside from the constitutive compounds implicated in fungal resistance of seeds, compounds induced by fungal attack may also be involved. It will improve our knowledge on seed defense to investigate the induced seed metabolites in infected seeds at different infection stages.

5.4 Biocontrol agents and seeds in seed banks

Seeds in seed banks interact not only with pathogens, but also with beneficial microbes. In this research, two *Trichoderma* species (*T. harzianum* and *T. hamatum*) were isolated from Arizona. *Trichoderma* spp. have been known as biocontrol agents for decays. However, it is unknown whether *Trichoderma* spp. can protect *N. attenuata* seeds from pathogen attack. In this study, the inhibition ability of *T. harzianum* on the native fungal pathogens was tested *in vitro*. *T. harzianum* can significantly inhibit the growth of the two fungal pathogens used in this study (Fig. 15). Additionally, there were more pathogenic fungal species isolated from Utah where no *Trichoderma* was found, than from Arizona. Likely, *Trichoderma* spp. in Arizona limit the diversity of culturable pathogenic fungi. More detailed research should be performed before the conclusion is drawn. It is possible that *N. attenuata* seeds in native seed banks might interact with and recruit native *Trichoderma* spp. to protect themselves from pathogens. To test this hypothesis, the following two assays should be conducted. Firstly, seeds can be treated with *Trichoderma* spores before they are incubated with pathogens. If the seeds pretreated

with *Trichoderma* spores are more viable than seeds receiving a mock pre-treatment, it can be concluded that *Trichoderma* can protect seeds *in vitro*. However, the native seed bank is a more complex environment. Hubbard *et al.* (1983) found that a lab-effective *T. hamatum* strain was ineffective to reduce plant disease when applied on seeds planted in field soils where microbes that compete for iron were present. Therefore, secondly, field assay should be performed in the natural habitats. Seeds treated with *Trichoderma* spores, and mock-treated seeds, should be buried in the field for at least a year, and then their viability should be quantified.

The findings in this study also raised another question: Do different metabolites of seeds or different paternities confer seeds with different abilities to interact with beneficial microbes? Many species in the genus *Trichoderma* have been shown to serve as biocontrol agents since the 1930s (Weindling, 1932), but only recently has the interaction between plants and *Trichoderma* drawn more attention. It has been discovered that *Trichoderma* can infect and colonize plant roots and establish chemical communication with host plants, and the infection can induce resistance in plants (Yedidia *et al.*, 1999; Harman *et al.*, 2004). Once plants are infected by *Trichoderma*, it functions as a mycorrhizal fungus and provides benefits to plants (Harman, 2000). More essentially, the colonization of *Trichoderma* can strongly influence the host plants by altering their gene expression in not only roots, but also shoots (Djonovic *et al.*, 2007; Shoresh and Harman, 2008; Shoresh *et al.*, 2010; Bae *et al.*, 2011). The changes induced by *Trichoderma* generally improve plant performance and also activate plant systemic defense mechanisms. The activated innate defense systems of the plant can reduce diseases caused by fungi and bacteria (Harman *et al.*, 2004). It is possible that *Trichoderma* colonizes seeds and that this colonization is influenced by seeds' metabolite differences. Consequently, seeds with different paternities might gain different abilities to defend themselves against the local pathogens in seed banks. Further studies are required to test these hypotheses.

In this study, it was found that the weak pathogen (*F. acuminatum*) possessed a defense mechanism against *T. harzianum in vitro*, while the virulent pathogen (*Alternaria* sp.) was completely vulnerable. Meanwhile, *N. attenuata* seeds not infected by *Trichoderma* spp. are

resistant to *F. acuminatum*, but susceptible to the *Alternaria* sp. *in vitro*. Perhaps, seeds of *N. attenuata* in the native seed bank (Arizona) often encounter *F. acuminatum*, but confront *Alternaria* sp. less commonly. Since *F. acuminatum* is resilient to *T. harzianum*, whereas *Alternaria* sp. is vulnerable to *T. harzianum*, it is likely that *F. acuminatum* is more widely distributed in the soil, while the distribution of *Alternaria* sp. is limited. Therefore, *N. attenuata* seeds might have evolved defense mechanisms against *F. acuminatum* but less resilience to *Alternaria* sp.. Further research may focus on seed-*Trichoderma*-pathogen interactions to understand the mechanisms of seed defense in *N. attenuata*.

5.5 Outlook

The findings in this thesis indicate that genetically different *N. attenuata* paternal plants impart their offspring seeds with diverse seed metabolomes. And together with other seed traits not measured here, it is possible that different paternities lead to different seed performance in the seed bank. In this study, the findings are not sufficient to demonstrate if paternities influence seed performance, because this study only artificially chose a few hybrids of seeds and tested their fungal resistance in lab against a few fungal species. The conditions *in vitro* are much simpler than the conditions *in situ*. In nature, it has been estimated that there are about 1.5 million species of fungi, in total, but only about 70,000 species have been described (Borneman and Hartin, 2000). It has been also estimated that 99% of all microbial species are unclassified or uncharacterized, owing to the difficulties in culturing most of microorganisms for detailed studies (Torsvik *et al.*, 1990; Ward *et al.*, 1990; Rondon *et al.*, 1999). The full diversity, functional types, and the ecological relationships among soil microorganisms remain poorly understood (Rondon *et al.*, 1999; Smit *et al.*, 1999; Sessitsch *et al.*, 2001; Smit *et al.*, 2001).

Besides, seed performance is not only determined by the seeds’ interaction with microbes and the seed susceptibility to pathogens. It includes all characteristics and traits that influence the seed longevity, since any of these may influence viability and reproductive potential of the offspring. And these traits may be important even before the seeds enter the seed bank.

Corimelaena extensa (Hemiptera, Corimelaenidae) is a seed-feeder on *N. attenuata* prior to seed dispersal (Stanton *et al.*, 2016). Feeding of *C. extensa* on *N. attenuata* seeds strongly decreases seed viability in the seed bank (Stanton *et al.*, 2016). Stanton *et al.* (2016) inferred that the feeding of this insect may increase the rate of microbial attack in the seed bank. It is also possible that this insect selectively feeds on seeds with certain traits, such as metabolites that confer seeds with greater longevities in the seed bank. Do different paternities influence seed defense against seed predators before seeds enter the seed bank? More research is required to unveil the answer.

It is possible that interactions with microbes are not the principal factors that affect seed longevity in seed banks after seeds are dispersed. On one hand, post-dispersal seed predation by insects, rodents, birds etc. is potentially an important factor that affects seed mortality, and thus influences seed bank dynamics in many ecosystems (Crawley, 1989; Morgan, 1995; Cardina *et al.*, 1996). On the other hand, innate seed traits that influence seed qualities, such as size, metabolism, dormancy, etc., can affect seed longevity as well. For example, the major causes of seed natural ageing and mortality are related to lipid peroxidation, peroxide-scavenging enzyme inactivation or protein degradation, disruption of cellular membranes, and damage to genetic integrity (Walters, 1998; McDonald, 1999; Goel and Sheoran, 2003). All these metabolites and metabolism may regulate seed longevity.

In order to understand the relationship among nonrandom mating, paternal influences and the performance of the offspring seeds, it is crucial to ascertain the main factors that affect the seed performance in native habitats. However, paternal influences on their offspring may not merely be traits of seeds. Adaptation of seedlings, adult plants, and reproductive ability are also important aspects of offspring fitness. Some paternal genotypes may improve offspring fitness at the post-germination stage of their life cycles. Absolutely superior paternal genotypes cannot exist, because if so, inferior paternal genotypes would have been wiped out by evolution. Since diploid offspring carry both sets of chromosomes from the parents, offspring fitness is affected not only by paternity, but also by maternity (Antonovics and Schmitt, 1986; Pires *et al.*, 2016). It

is likely that one paternal genotype has different influences on its offspring when combined with different maternal genotypes. If a paternal genotype can produce better offspring on a maternal genotype, does the paternal genotype also produce successful pollen when competing with other pollen donors on the maternal genotype in the process of nonrandom mating? More studies are required to investigate the paternal influence on the offspring fitness, and the linkage between nonrandom mating and offspring fitness in *N. attenuata*.

6. Summary

In the late 1970s, 100 years after Darwin (1871) proposed the theory of sexual selection, the theory was applied in plants (Willson, 1979). In many plant species, seeds are often sired disproportionately to the relative amounts of pollen from different donors initially deposited on the stigma. Bhattacharya and Baldwin (2012) found that, in *Nicotiana attenuata*, after equal amounts of pollen from genetically different wild donors were simultaneously loaded onto the stigma, maternal plants (UtWT, Utah, USA) selected for self pollen in binary mixed pollinations. Furthermore, between two cross pollen donors (G2 and G10, Utah, USA), UtWT maternal plants selected less strongly against G2 than G10, when binary mixed pollinations of self and cross pollen were manually conducted. It is elusive that if the *in vivo* pollen tube (PT) growth rates of different pollen donors are involved in the nonrandom mating in *N. attenuata*. Bhattacharya and Baldwin (2012) also demonstrated that ethylene-deficient (irACO, silenced in 1-aminocyclopropane-1-carboxylic acid oxidase activity) and ethylene-insensitive (ETR1, overexpressing a mutated ethylene receptor) transgenic lines lost the ability of selection, *i.e.* the seeds on these lines were sired proportionately to the pollen used for mixed pollination. Therefore, these two maternal lines were used as controls in this study. Aside from ethylene, it is unknown if stylar secondary metabolites are involved in the nonrandom mating. Moreover, since many studies revealed that paternity may affect the quality of offspring, this study also tried to test if different paternal genotypes can impact on metabolomes and pathogen resistance of their offspring seeds.

To demonstrate that the differential growth rates of PTs from different donors are involved in nonrandom seed siring in *N. attenuata*, it is important to view the growths of competitive PTs *in vivo* in a same style. Since previous study showed that genetically transformed pollen may affect the pattern of nonrandom mating in *N. attenuata* (Bhattacharya and Baldwin, 2012), pollen used in this study should not be transformed with fluorescence. This study firstly provided a novel method to visualize the *in vivo* differential PT growth rates of two wild competitive pollen donors in the same style. Using different pollen pairs (UtWT+G2 or UtWT+G10) on UtWT maternal plants, it was discovered that the non-self PT (G2 or G10) was

constantly shorter than self PT (UtWT) *in vivo*. However, in non-selective maternal styles (irACO and ETR1 lines), the PT length of G10 was shorter, equal or longer than UtWT. Moreover, the length difference between G10 and UtWT in irACO and ETR1 styles was significantly reduced compared with the length difference in UtWT styles. In addition, the difference between the non-favored non-self pollen donor G10 and UtWT was significantly larger than the difference between favored non-self pollen donor G2 and UtWT in UtWT styles. Therefore, it was demonstrated that the differential *in vivo* PT growth rates were involved in nonrandom mating. The *in vivo* PT growth rate was correlated with the seed siring in *N. attenuata*. It was revealed that 5 *O*-acyl sugars from styles may be involved in mate selection process in *N. attenuata*, by applying comparisons between the metabolomic profiles (non-targeted LC-MS) of UtWT styles pollinated with the two different pollen pairs (UtWT+G10 and UtWT+G2), and between the metabolomic profiles of the selective (UtWT) and non-selective (irACO) styles pollinated with the same pollen combination (UtWT+G10).

In this study, the influence of paternal genotypes on their offspring seeds was investigated. In addition to the UtWT maternal genotype, 11 natural genotypes collected from Utah native populations were used as paternal lines. Similarly, on an AzWT (Arizona, USA) maternal genotype, 15 native genotypes collected from Arizona populations were used as paternal genotypes. Metabolomics analyses of seeds indicated that different paternal lines imparted their hybrid offspring seeds with distinct metabolomes. In order to test if the different paternities may confer the seeds with different pathogen resistance, 70 fungal isolates were obtained from seeds buried in Utah and Arizona for further studies. Two fungal species isolated from Arizona were identified as seed pathogens of *N. attenuata* and used for later research. Four different offspring seeds from four Arizona paternal lines were used in fungal bioassays. One of the seed pathogens (*Fusarium acuminatum*) caused different mortalities in offspring seeds. It suggested that Arizona paternal lines might confer their offspring seeds with different abilities of fungal pathogen resistances. Data analyses on seed metabolomes showed that 22 features may be involved in the differential *F. acuminatum* resistances of the *N. attenuata* seeds. Aside from pathogens, two biocontrol agents (*Trichoderma*) were isolated from the

natural habitat (Arizona) as well. Further studies are needed to demonstrate if seeds with different genetic and metabolomic background can interact with them differently against pathogens.

This study has provided a novel method to track *in vivo* differential PT growth and the secondary metabolites potentially involved in nonrandom mating. These findings will help to improve our understanding on the mechanism of pre-zygotic nonrandom mating in *N. attenuata*. The seed metabolites that might be involved in pathogen resistance discovered in this study may open a new direction for further studies to improve our knowledge of seed-microbe interaction, seed defense and seed longevity. It is reasonable to infer *N. attenuata* might leverage nonrandom mating to allow beneficial pollen donors to sire more seeds with traits improving adaptation in native seed banks. However, since the nonrandom mating pattern of the 15 Arizona paternal lines in AzWT maternal line is still unclear, it is impossible to link nonrandom mating with the differential fungal resistances of seeds. Besides, *in vitro* bioassays of fungus-seed interaction are not sufficient to predict the performances of the seeds *in situ*. Further seed burial experiments in the natural habitats are required to demonstrate paternal influences on offspring seed performance in seed banks. The discoveries in this study open the path to continue investigating if mate selection of *N. attenuata* can improve the fitness of offspring.

7. Zusammenfassung

In den späten 1970er Jahren, 100 Jahre nachdem Darwin (1871) die Theorie der sexuellen Selektion vorgeschlagen hatte, wurde die Theorie auf Pflanzen angewendet (Willson, 1979). Bei vielen Pflanzenarten werden Samen oft unproportional zu den relativen Mengen an Pollen von verschiedenen Spendern, die anfänglich auf dem Stigma abgelagert wurden, befruchtet. Bhattacharya und Baldwin (2012) fanden heraus, dass in *Nicotiana attenuata* mütterliche Pflanzen (UtWT, Utah, USA) in binären Mischbestäubungen nach Selbstpollen selektierten, nachdem gleiche Mengen Pollen von genetisch unterschiedlichen Wildspendern gleichzeitig auf das Stigma geladen wurden. Darüber hinaus selektierten zwischen zwei Kreuzpollenspendern (G2 und G10, Utah, USA) die maternalen UtWT-Pflanzen weniger stark gegen G2 als gegen G10, wenn binäre Mischbestäubungen von Eigen- und Kreuzpollen manuell durchgeführt wurden. Es ist bislang schwer fassbar, ob die *in-vivo*-Pollenschlauch (PS) -Wachstumsraten verschiedener Pollenspender an der nicht-zufälligen Paarung in *N. attenuata* beteiligt sind. Bhattacharya und Baldwin (2012) zeigten auch, dass Ethylen-defiziente (irACO, Stillegung von 1 Aminocyclopropan-1-carbonsäure-Oxidase-Aktivität) und Ethylen-insensitive (ETR1, Überexpression des Ethylenrezeptors) transgene Linien die Fähigkeit zur Selektion verloren haben. Die Samen dieser Linien wurden proportional zu den Pollen in der gemischten Bestäubung befruchtet. Daher wurden diese beiden mütterlichen Linien als Kontrollen in dieser Studie verwendet. Abgesehen von Ethylen ist nicht bekannt, ob Sekundärmetabolite des Griffels an der nicht-zufälligen Paarung beteiligt sind. Da viele Studien ergaben, dass die Vaterschaft die Qualität der Nachkommen beeinflussen kann, wurde in dieser Studie auch getestet, ob verschiedene väterliche Genotypen sich auf Metabolom und Pathogenresistenz ihrer Nachkommen auswirken können.

Um zu demonstrieren, dass die unterschiedlichen Wachstumsraten von PSs von verschiedenen Spendern an der nicht-zufälligen Samenbildung in *N. attenuata* beteiligt sind, ist es wichtig, das Wachstum konkurrierender PSs *in vivo* im gleichen Griffel zu betrachten. Da frühere Studien zeigten, dass genetisch transformierte Pollen das Muster der nicht-zufälligen Paarung in *N. attenuata* beeinflussen können (Bhattacharya und Baldwin, 2012), sollte der in

dieser Studie verwendete Pollen nicht mit Fluoreszenz transformiert werden. Diese Studie lieferte zum ersten Mal einen neue Methode um die unterschiedlichen PS-Wachstumsraten von zwei wilden, konkurrierenden Pollenspendern *in vivo* im selben Griffel zu visualisieren. Unter Verwendung von verschiedenen Pollenpaaren (UtWT + G2 oder UtWT + G10) auf UtWT-Mutterpflanzen wurde festgestellt, dass der Nicht-Selbst-PS (G2 oder G10) *in vivo* konstant kürzer als der Selbst-PS (UtWT) war. In nichtselektiven maternalen Formen (irACO- und ETR1-Linien) war die PS-Länge von G10 jedoch kürzer, gleich oder länger als die von UtWT. Darüber hinaus war der Längenunterschied zwischen G10 und UtWT in den Griffeln von irACO und ETR1 im Vergleich zum Längenunterschied in den UtWT-Griffeln signifikant reduziert. Außerdem war der Unterschied zwischen dem nicht favorisierten Nicht-Eigenpollen-Spender G10 und UtWT signifikant größer als der Unterschied zwischen dem bevorzugten Nicht-Eigenpollen-Spender G2 und UtWT in UtWT-Griffeln. Damit wurde gezeigt, dass die unterschiedlichen *in vivo* PS-Wachstumsraten an der nicht-zufälligen Paarung beteiligt sind. Die *in vivo* PS-Wachstumsrate korrelierte mit der Befruchtung der Samen in *N. attenuata*. Es wurde gezeigt, dass 5 O-Acyl-Zucker aus Griffeln am Prozess der Paarung in *N. attenuata* beteiligt sein könnten, indem zwischen den metabolomischen Profilen (non-targeted LC-MS) von mit den zwei verschiedenen Pollenpaaren bestäubten UtWT-Arten (UtWT + G10 und UtWT + G2) und zwischen den metabolomischen Profilen der selektiven (UtWT) und nichtselektiven (irACO) Linien, die mit der gleichen Pollenkombination bestäubt wurden (UtWT + G10), verglichen wurde.

In dieser Studie wurde der Einfluss väterlicher Genotypen auf ihre Nachkommensamen untersucht. Zusätzlich zum maternalen UtWT-Genotyp wurden 11 natürliche Genotypen aus in Utah beheimateten Populationen als väterliche Linien verwendet. In ähnlicher Weise wurden auf einem mütterlichen Genotyp von AzWT (Arizona, USA) 15 aus Arizona-Populationen gewonnene native Genotypen als väterliche Genotypen verwendet. Metabolomische Analysen von Samen zeigten, dass verschiedene väterliche Linien ihren hybriden Nachkommensamen mit unterschiedlichen Metabolomen weitergaben. Um zu testen, ob die verschiedenen Väter den Samen eine unterschiedliche Pathogenresistenz verleihen können, wurden 70 Pilzarten aus in Utah und Arizona vergrabenen Samen für weitere Studien isoliert. Zwei aus Arizona isolierte

Pilzarten wurden als Samenpathogene von *N. attenuata* identifiziert und für spätere Untersuchungen verwendet. Vier verschiedene Nachkommensamen aus vier väterlichen Linien von Arizona wurden in Pilz-Bioassays verwendet. Einer der Samenpathogene (*Fusarium acuminatum*) verursachte unterschiedliche Sterblichkeit in Nachkommensamen. Die Ergebnisse legten nahe, dass die väterlichen Linien aus Arizona ihren Nachkommensamen unterschiedliche Resistenz gegenüber pathogenen Pilzen verleihen können. Datenanalysen an Samenmetabolomen zeigten, dass 22 Merkmale an den unterschiedlichen Resistenzen der *N. attenuata* Samen gegenüber *F. acuminatum* beteiligt sein könnten. Neben Krankheitserregern wurden auch zwei biologische Bekämpfungsmittel (*Trichoderma*) aus dem natürlichen Lebensraum (Arizona) isoliert. Weitere Studien sind erforderlich um zu zeigen, ob Samen mit unterschiedlichem genetischen und metabolischen Hintergrund diese anders gegen Pathogene einsetzen können.

Diese Studie stellt eine neue Methode zur Verfolgung des *in vivo* differentiellen PT-Wachstums und der Sekundärmetaboliten, die möglicherweise an nicht-zufälliger Paarung beteiligt sind, bereit. Die Ergebnisse werden dazu beitragen, unser Verständnis des Mechanismus der präzygotischen nicht-zufälligen Paarung in *N. attenuata* zu verbessern. Die Samenmetaboliten, die möglicherweise an der Pathogenresistenz beteiligt sind, die in dieser Studie entdeckt wurden, könnten eine neue Richtung für weitere Studien eröffnen und unser Wissen über Samen-Mikroben-Interaktion, Samenabwehr und Samen-Langlebigkeit verbessern. Man könnte daraus zu folgern, dass *N. attenuata* über die nicht-zufällige Paarung nützlichen Pollenspendern ermöglicht, mehr Samen mit Merkmalen zu vererben, die die Anpassung in nativen Samenbanken verbessern. Da jedoch das nicht-zufällige Paarungsmuster der 15 väterlichen Linien von Arizona in der mütterlichen AzWT-Linie noch unklar ist, ist es unmöglich, die nicht-zufällige Paarung mit den unterschiedlichen Pilzresistenzen von Samen zu verknüpfen. Darüber hinaus sind *in-vitro*-Bioassays der Pilz-Samen-Wechselwirkungen nicht ausreichend, um die Leistung der Samen *in-situ* vorherzusagen. Weitere Samen-Vergrabungsexperimente in den natürlichen Lebensräumen sind erforderlich, um väterliche Einflüsse auf die Tüchtigkeit der Nachkommen in Samenbanken nachzuweisen. Die Entdeckungen in dieser Studie öffnen den

Weg für weitere Untersuchungen darüber, ob die Partnerwahl von *N. attenuata* die Fitness von Nachkommen verbessern kann.

8. Supplementary data

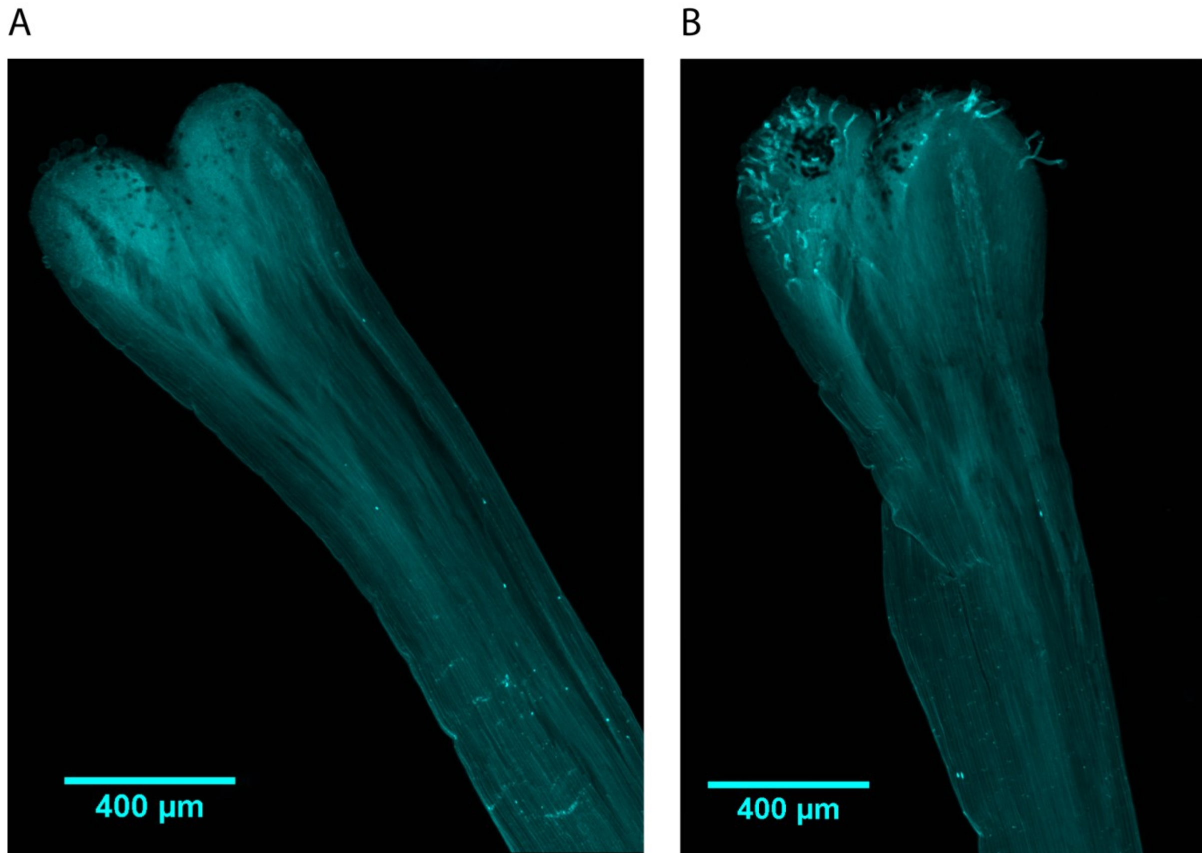
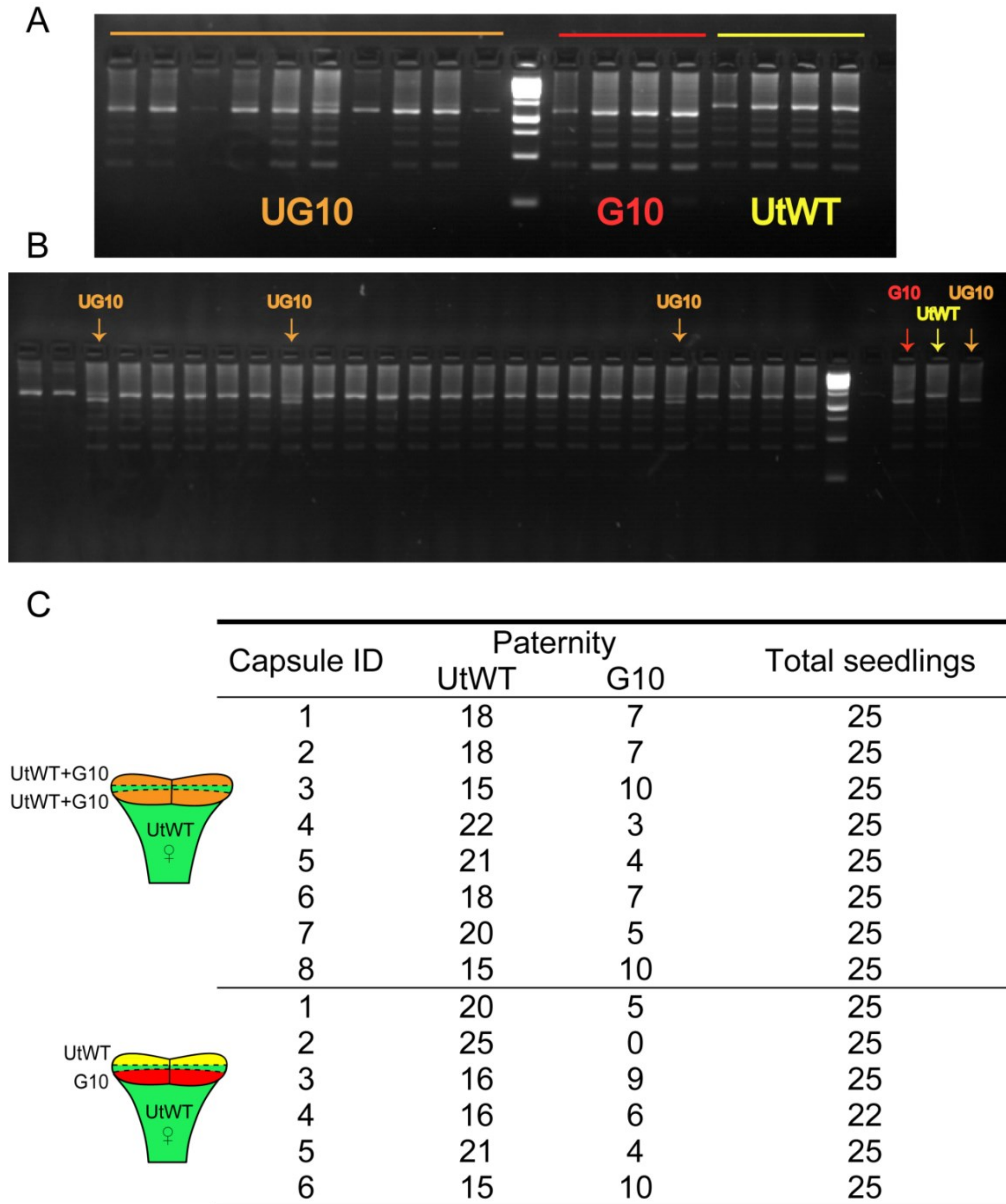


Figure S1. Representative images of samples with no germinated pollen and samples harvested at 1 h post-pollination. A) Samples with no germinated pollen. B) The picture shows short pollen tubes (PTs) at 1 h after pollination.



Capsule ID	Paternity UtWT	Paternity G10	Total seedlings
1	18	7	25
2	18	7	25
3	15	10	25
4	22	3	25
5	21	4	25
6	18	7	25
7	20	5	25
8	15	10	25
1	20	5	25
2	25	0	25
3	16	9	25
4	16	6	22
5	21	4	25
6	15	10	25

Figure S2. Results of seedling genotyping. A) Band patterns of seedlings with known genotypes. B) Representative gel picture of seedlings from one capsule. C) Paternity of capsules obtained from two different pollination methods.

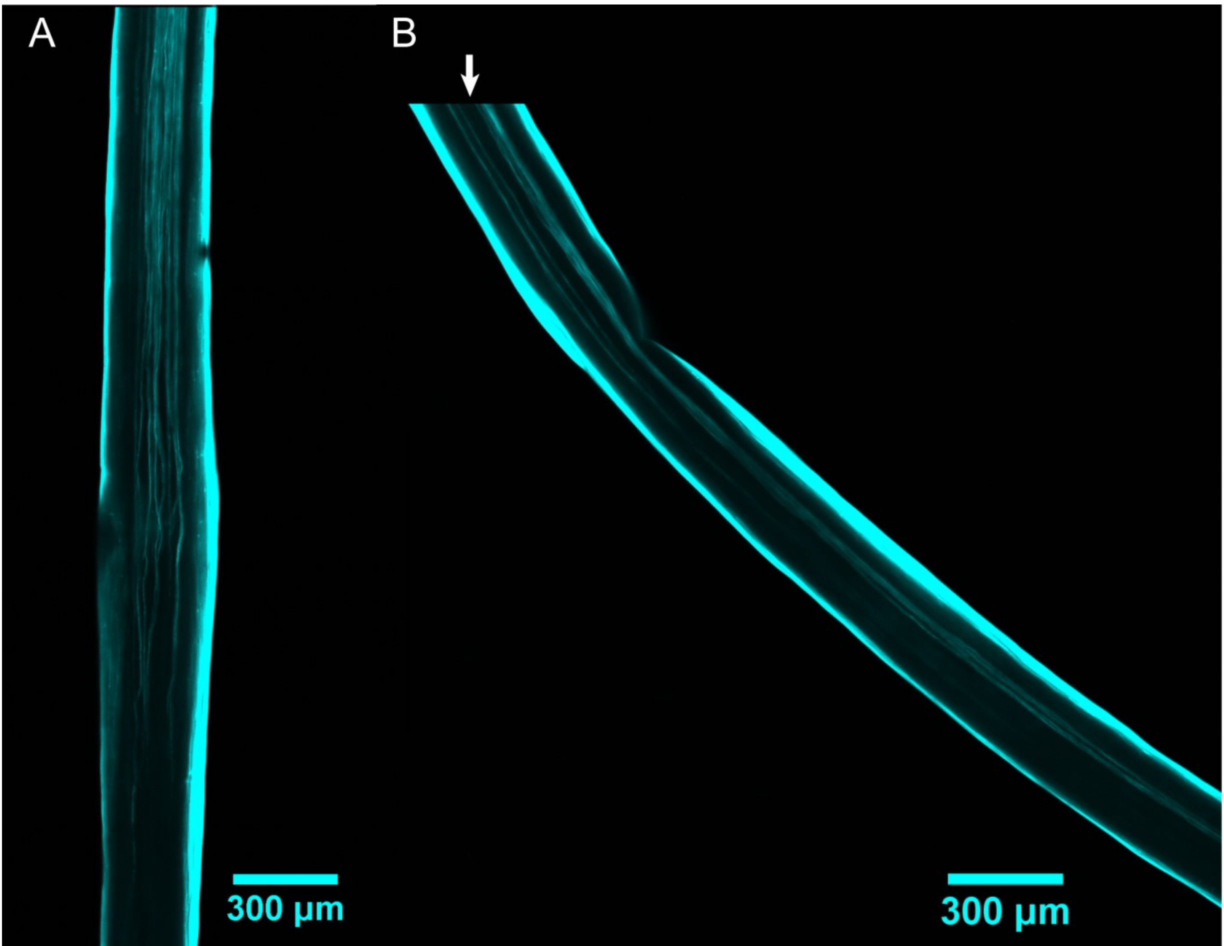


Figure S3. Representative images of samples with unclear gaps and clear gaps between two PT bundles *in vivo*. A) Representative image of an UtWT style harvested 8 h after being pollinated with UtWT+G2 separately on the two transmitting zones. PTs from two pollen donors mixed in the styles 8 h after pollination, and no clear gap can be observed. B) Representative image of an UtWT style harvested 8 h after being pollinated with UtWT+G10 separately on the two transmitting zones. PTs from two pollen donors grew separately in the styles 8 h after pollination, and a clear gap indicated with a white arrow can be observed.

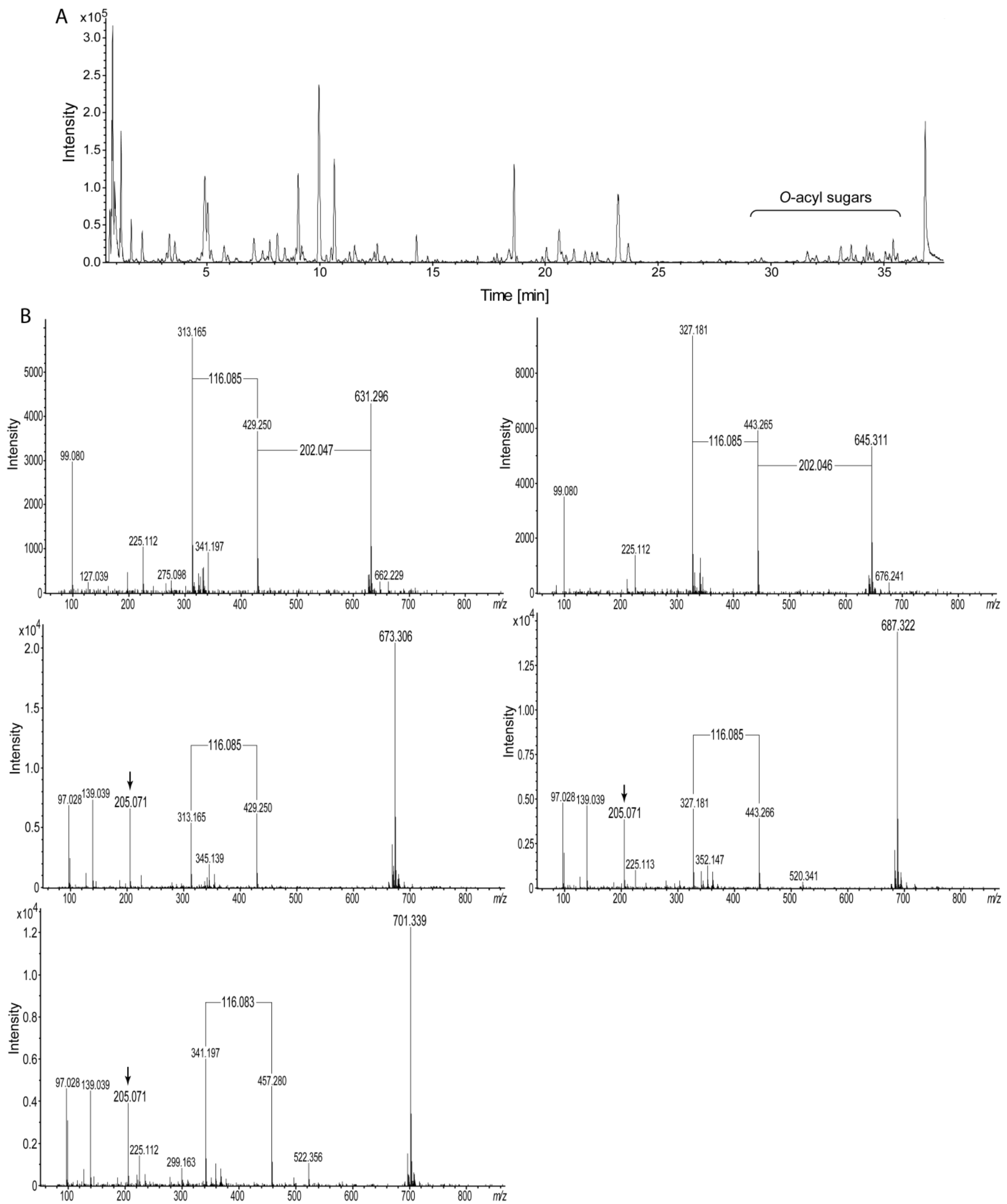


Figure S4. Example chromatogram (A) of the extract from 5 complete *N. attenuata* styles (with stigmas) and mass spectra of the potentially interesting *O*-acyl sugars (B).

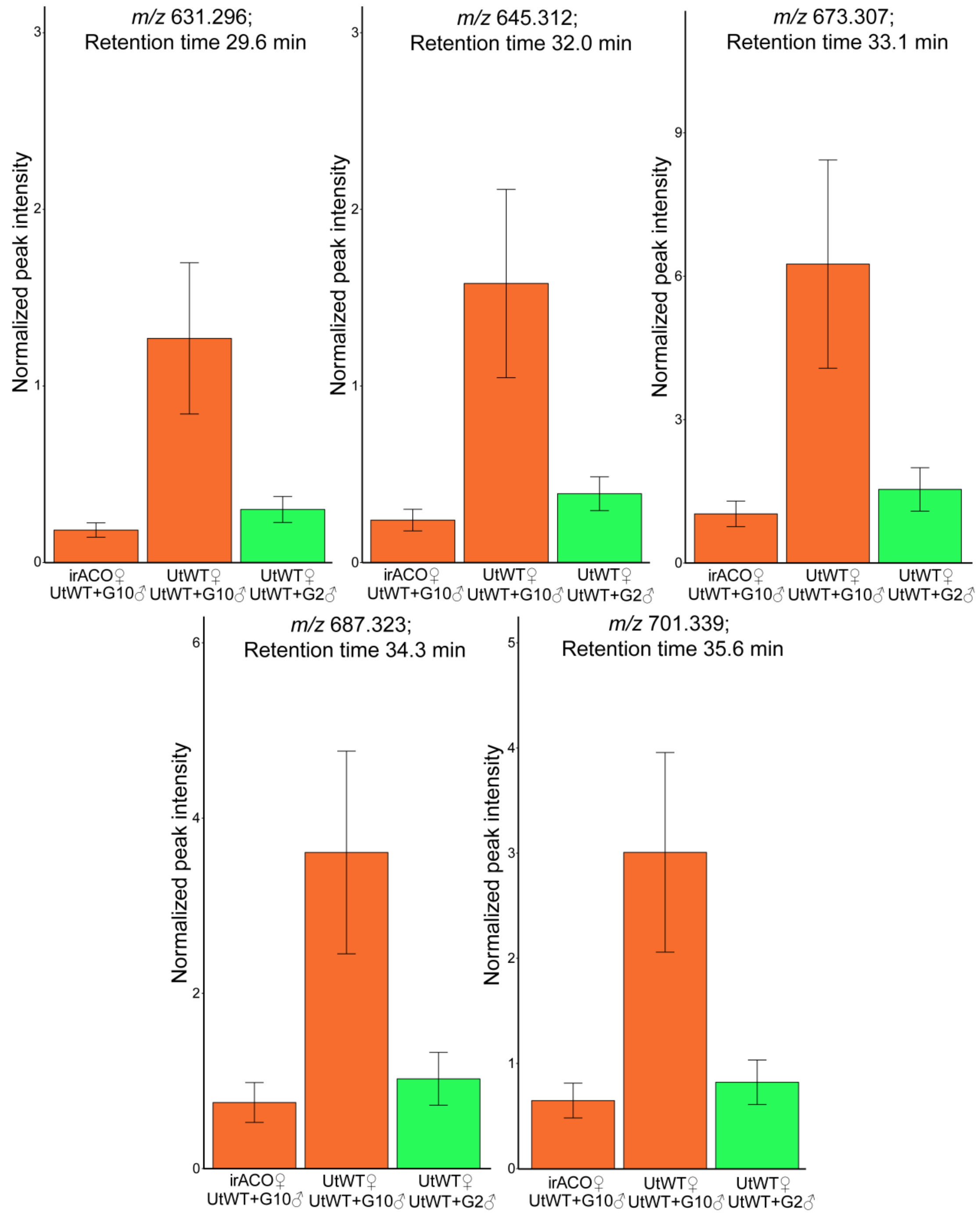


Figure S5. Bar plots of the 5 parent ions of the *O*-acyl sugars. Peak intensities were normalized by 75 percentile normalization.

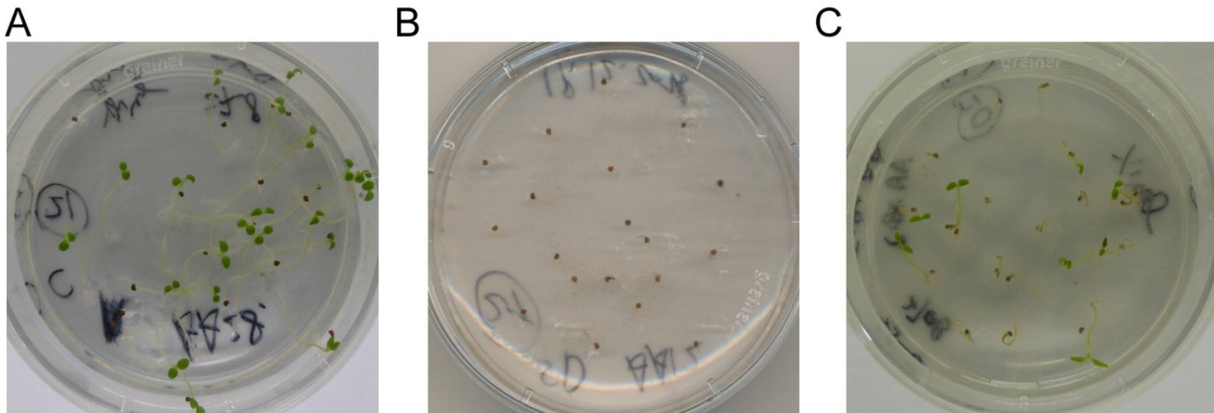


Figure S6. Representative pictures of the germinated seedlings after bioassay. A) Seeds with control treatment, *i.e.* PDA treatment. B) Seeds treated with *Alternaria* sp.. C) Seeds treated with *Fusarium acuminatum*.

Table S1. Numbers of styles used for each PT-length data set of different pollination combinations

Maternal genotype	Paternal genotype	Time point (h)	Number of samples with PTs	Number of samples without germinated pollen	Number of samples with unclear gap
UtWT	UtWT	1	4	14	NA
UtWT	G2	1	7	11	NA
UtWT	G10	1	9	8	NA
UtWT	UtWT	4	12	3	NA
UtWT	G2	4	15	3	NA
UtWT	G10	4	14	1	NA
UtWT	UtWT	8	17	1	NA
UtWT	G2	8	17	2	NA
UtWT	G10	8	27	2	NA
UtWT	UtWT+G10	4	10	NA	0
UtWT	UtWT+G2	4	9	NA	1
ETR1	UtWT+G10	4	10	NA	0
irACO	UtWT+G10	4	9	NA	0
UtWT	UtWT+G10	8	21	NA	3
UtWT	UtWT+G2	8	7	NA	15
ETR1	UtWT+G10	8	11	NA	9
irACO	UtWT+G10	8	9	NA	7

NA, data were not collected.

Table S2. ITS sequences of the selected 3 fungal species used in this study.

Fungal species	ITS sequence
Alternaria sp.	ACGCCGCATCCTAGCAGANGCGCGGACCTCAGTCCAGGCTGGTAGTATGTCGTCTCCCCTATAAGGCCTCCCCGAAAGGAGGTACGTGACAGAGACCTTTATCCTACCGCCC AAACTGATGCTGGCCTGCCTACAGAAGAGTGCACCGGGTAGAAACCCGGATGAGCAACTGTAAGCAAGTCTGGCTGCAAGCGCTTCCCTATCAACAATTTNACGTGCTGTTT GACTCTCTTTCCAAAGTGCTTTTCATCTTTCGATCACTCTACTTGTGCGCTATCGGTCTCTGGCCAATATTTAGCTTTAGAAGAAATGTACCTCCCATTTAGAGCTGCATTCCCA AACAACTCGACTCGTCGAAGGGGCTTTACACGGCAATAGCCAGCGACCACGTACGGGATTCTCACCCTCTGTGACGTCCTGTTCCAAGGAACTTGGACCGCTGCCAAAGCCA AAGCGCCCTCTGCAAATTACAACTCGGACTCTAAAAGAGCCAGATTTCAAATTTGAGCTGTTGCCGCTTCACTCGCCGTTACTAGGGCAATCCCTGTTGGTTTCTTTTCCTCCG CTTATTGATATGCTTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCAAAAGTTGAAAAAAAGGCTTAATGGATGCTAGACCTTTGCTGATAGAGAGTGCGACTTGTGCT GCGCTCCGAAACCAGTAGGCCGGCTGCCAATTACTTTAAGGCGAGTCTCCAGCAAAGCTAGAGACAAGACGCCCAACACCAAGCAAAGCTTGAGGGTACAAATGACGCTCG AACAGGCATGCCCTTTGGAATACCAAAGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACACTACTTATCGCATTTCGCTGCGTTCTTCATCGAT G
Fusarium acuminatum	TTTGGTCATTAGAGGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTTACAACTCCCAAACCCCTGTGAACATACCTTAATGTTG CCTCGGCGGATCAGCCCGCGCCCCGTAAAACGGGACGGCCCGCCAGAGGACCCAAACTCTAATGTTTCTTATTGTAACTTCTGAGTAAAACAAACAAATAAATCAAAACTTTC AACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCG CTGGTATTCCGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCCCGGGTTTGGTGTTGGGGATCGGCTCTGCCCTTCTGGGCGGCGCCGCCCCCGAAATACAT TGGCGGTCTCGCTGCAGCCTCCATTGCGTAGTAGCTAACACCTCGCAACTGGAACGCGGCGCGGCCATGCCGTAAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAG GAATACCCGG
Trichoderma harzianum	ATCCTTGCAGANGCGCGGTCCTCAGTCCACCGCAGGGTATTATGCAACGGGCTATAACACTCCCGGAGGAGCCACGTTCCCGAAGCCTTTTTCCCCCGCGACGAACTGATGC TGGCCTAGACGCGGCGAAGTGCACCGGAGAGAACCCCGGATGATCCGCCGCGCCCAAGTCTGGTCACAAGCGCTTCCCTTTCAACAATTTCACGTACTATTTAACCCTCTTTT CAAGGTGCTTTTCATCTTTCGATCACTCTACTTGTGCGCTATCGGTCTCTGGCCAATATTTAGCTTTAGAAGACATATACCTCCCATTTTGAGCAGCATTCCCAAACTACTCGAC TCGTCGAAGGAGCTTTACAGAGGCTCGGCGGCCAGCCAGACGGGGCTCTCACCCTCTGTGGCGTCCCGTTCCAGGGAACTCGGGCGGCACCTCACCAAAAGCATCCTCTAC AAATTACAACTCGGGCCCTAGGGACCAGATTTCAAATTTGAGCTGTTGCCGCTTCACTCGCCGTTACTGGGGCAATCCCTGTTGGTTTCTTTTCCTCCGCTTATTGATATGCTTA AGTTCAGCGGGTATTCCTACCTGATCCGAGGTCAACATTTCAGAAGTTGGGTGTTTAACGGCTGTGGACGCGCCGCGCTCCCGATGCGAGTGTGCAAACTACTGCGCAGGA GAGGCTGCGGCGAGACCGCCACTGTATTTCGGAGACGGCCACCCGCTAAGGGAGGGCCGATCCCCAACGCCGACCCCCCGGAGGGGTTCGAGGGTTGAAATGACGCTCGG ACAGGCATGCCCGCC

Table S3. The number of pollen grains on one transmitting zone of the stigma in separated pollination.

Stigma ID	Number of pollen grain
1	718
2	738
3	1218
4	706
5	957
6	717
7	736
8	791

9. References

Aizen M.A., Searcy K.B., Mulcahy D.L. (1990). Among-flower and within-flower comparisons of pollen tube growth following self-pollinations and cross-pollinations in *Dianthus chinensis* (Caryophyllaceae). *American Journal of Botany*. **77**, 671-676.

Anderson R.C., Liberta A.E., Packheiser J., Neville M.E. (1980). Inhibition of selected fungi by bacterial isolates from *Tripsacum dactyloides*. *Plant and Soil*. **56**, 149-152.

Andersson S. (1990). Paternal effects on seed size in a population of *Crepis tectorum* (Asteraceae). *Oikos*. **59**, 3-8.

Antonovics J., Schmitt J. (1986). Paternal and maternal effects on propagule size in *Anthoxanthum odoratum*. *Oecologia*. **69**, 277-282.

Armitage E.G., Godzien J., Alonso-Herranz V., López-Gonzálvez Á., Barbas C. (2015). Missing value imputation strategies for metabolomics data. *Electrophoresis*. **36**, 3050-3060.

Arnold S.J. (1983). Sexual selection: the interface of theory and empiricism. In: Bateson P., ed. *Mate choice*. Cambridge, Cambridge University Press.

Arnold S.J. (1994a). Bateman principles and the measurement of sexual selection in plants and animals. *American Naturalist*. **144**, S126-S149.

Arnold S.J. (1994b). Is there a unifying concept of sexual selection that applies to both plants and animals? *American Naturalist*. **144**, S1-S12.

Aronen T., Nikkanen T., Harju A., Tiimonen H., Haggman H. (2002). Pollen competition and seed-siring success in *Picea abies*. *Theoretical and Applied Genetics*. **104**, 638-642.

Arrendale R.F., Severson R.F., Sisson V.A., Costello C.E., Leary J.A., Himmelsbach D.S., van Halbeek H. (1990). Characterization of the sucrose ester fraction from *Nicotiana glutinosa*. *Journal of Agricultural and Food Chemistry*. **38**, 75-85.

Axelrood P.E., Neumann M., Trotter D., Radley R., Shrimpton G., Dennis J. (1995). Seedborne *Fusarium* on Douglas-fir: pathogenicity and seed stratification method to decrease *Fusarium* contamination. *New Forests*. **9**, 35-51.

Bae H., Roberts D.P., Lim H.S., Strem M.D., Park S.C., Ryu C.M., Melnick R.L., Bailey B.A. (2011). Endophytic *Trichoderma* isolates from tropical environments delay disease onset and induce resistance against *Phytophthora capsici* in hot pepper using multiple mechanisms. *Molecular Plant-Microbe Interactions*. **24**, 336-351.

Bahulikar R.A., Stanculescu D., Preston C.A., Baldwin I.T. (2004). ISSR and AFLP analysis of the temporal and spatial population structure of the post-fire annual, *Nicotiana attenuata*, in SW Utah. *BMC Ecol*. **4**, 12.

Bedinger P.A., Broz A.K., Tovar-Mendez A., McClure B. (2017). Pollen-pistil interactions and their role in mate selection. *Plant Physiology*. **173**, 79-90.

Benjamini Y., Hochberg Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society, Series B, Methodological*. **57**, 289-300.

Bertin R.I., Sullivan M. (1988). Pollen interference and cryptic self-fertility in *Campsis radicans*. *American Journal of Botany*. **75**, 1140-1147.

Berto P., Belingheri L., Dehorter B. (1997). Production and purification of a novel extracellular lipase from *Alternaria brassicicola*. *Biotechnology Letters*. **19**, 533-536.

Bhattacharya S., Baldwin I.T. (2012). The post-pollination ethylene burst and the continuation of floral advertisement are harbingers of non-random mate selection in *Nicotiana attenuata*. *Plant Journal*. **71**, 587-601.

Bissett J. (1991). A revision of the genus *Trichoderma*. II. Infrageneric classification. *Canadian Journal of Botany*. **69**, 2357-2372.

Bookman S.S. (1984). Evidence for selective fruit production in *Asclepias*. *Evolution*. **38**, 72-86.

Borneman J., Hartin R.J. (2000). PCR primers that amplify fungal rRNA genes from environmental samples. *Applied and Environmental Microbiology*. **66**, 4356-4360.

Bubner B., Gase K., Baldwin I.T. (2004). Two-fold differences are the detection limit for determining transgene copy numbers in plants by real-time PCR. *Bmc Biotechnology*. **4**, 14.

Burgess L.W., Wearing A.H., Toussoun T.A. (1975). Surveys of *Fusaria* associated with crown rot of wheat in eastern Australia. *Australian Journal of Agricultural Research*. **26**, 791-799.

Caetano-Anollés G., Crist-Estes D.K., Bauer W.D. (1988). Chemotaxis of *Rhizobium meliloti* to the plant flavone luteolin requires functional nodulation genes. *Journal of Bacteriology*. **170**, 3164-3169.

Cardina J., Norquay H.M., Stinner B.R., McCartney D.A. (1996). Postdispersal predation of velvetleaf (*Abutilon theophrasti*) seeds. *Weed Science*. **44**, 534-539.

Carlson A.L., Telligman M., Swanson R.J. (2009). Incidence and post-pollination mechanisms of nonrandom mating in *Arabidopsis thaliana*. *Sexual Plant Reproduction*. **22**, 257-262.

Casey C.E., O'Sullivan O.B., O'Gara F., Glennon J.D. (1998). Ion chromatographic analysis of nutrients in seed exudate for microbial colonisation. *Journal of Chromatography A*. **804**, 311-318.

Céspedes R., González B., Vicuña R. (1997). Characterization of a bacterial consortium degrading the lignin model compound vanillyl-beta-D-glucopyranoside. *Journal of Basic Microbiology*. **37**, 175-180.

Chae K., Lord E.M. (2011). Pollen tube growth and guidance: roles of small, secreted proteins. *Annals of Botany*. **108**, 627-636.

Chambers J.C., MacMahon J.A. (1994). A day in the life of a seed: movements and fates of seeds and their implications for natural and managed systems. *Annual Review of Ecology and Systematics*. **25**, 263-292.

Charlesworth D. (1989). Why do plants produce so many more ovules than seeds. *Nature*. **338**, 21-22.

Chet I., Harman G.E., Baker R. (1981). *Trichoderma hamatum*: its hyphal interactions with *Rhizoctonia solani* and *Pythium* spp.. *Microbial Ecology*. **7**, 29-38.

Chortyk O.T., Pomonis J.G., Johnson A.W. (1996). Syntheses and characterizations of insecticidal sucrose esters. *Journal of Agricultural and Food Chemistry*. **44**, 1551-1557.

Chortyk O.T., Severson R.F., Cutler H.C., Sisson V.A. (1993). Antibiotic activities of sugar esters isolated from selected *Nicotiana* species. *Bioscience Biotechnology and Biochemistry*. **57**, 1355-1356.

Cocucci A.A., Marino S., Baranzelli M., Wiemer A.P., Sérsic A. (2014). The buck in the milkweed: evidence of male-male interference among pollinaria on pollinators. *New Phytologist*. **203**, 280-286.

Crawford B.C.W., Ditta G., Yanofsky M.F. (2007). The NTT gene is required for transmitting-tract development in carpels of *Arabidopsis thaliana*. *Current Biology*. **17**, 1101-1108.

Crawley M.J. (1989). Insect herbivores and plant population dynamics. *Annual Review of Entomology*. **34**, 531-564.

Cruzan M.B. (1986). Pollen tube distributions in *Nicotiana glauca*: evidence for density dependent growth. *American Journal of Botany*. **73**, 902-907.

Cruzan M.B. (1989). Pollen tube attrition in *Erythronium grandiflorum*. *American Journal of Botany*. **76**, 562-570.

Cruzan M.B. (1990). Pollen-pollen and pollen-style interactions during pollen tube growth in *Erythronium grandiflorum* (Liliaceae). *American Journal of Botany*. **77**, 116-122.

Cruzan M.B., Barrett S.C.H. (1993). Contribution of cryptic incompatibility to the mating system of *Eichhornia paniculata* (Pontederiaceae). *Evolution*. **47**, 925-934.

Cruzan M.B., Barrett S.C.H. (1996). Postpollination mechanisms influencing mating patterns and fecundity: an example from *Eichhornia paniculata*. *American Naturalist*. **147**, 576-598.

Currah L. (1981). Pollen competition in onion (*Allium cepa* L.). *Euphytica*. **30**, 687-696.

Darwin C. (1871). *The descent of man and selection in relation to sex*. London, UK: John Murray.

Deaker R., Roughley R.J., Kennedy I.R. (2004). Legume seed inoculation technology - a review. *Soil Biology & Biochemistry*. **36**, 1275-1288.

Dennis C., Webster J. (1971). Antagonistic properties of species-groups of *Trichoderma*: III. Hyphal interaction. *Transactions of the British Mycological Society*. **57**, 363-369.

Djonovic S., Vargas W.A., Kolomiets M.V., Horndeski M., Wiest A., Kenerley C.M. (2007). A proteinaceous elicitor Sm1 from the beneficial fungus *Trichoderma virens* is required for induced systemic resistance in maize. *Plant Physiology*. **145**, 875-889.

Druzhinina I.S., Seidl-Seiboth V., Herrera-Estrella A., Horwitz B.A., Kenerley C.M., Monte E., Mukherjee P.K., Zeilinger S., Grigoriev I.V., *et al.* (2011). *Trichoderma*: the genomics of opportunistic success. *Nature Reviews Microbiology*. **9**, 749-759.

Dunn O.J. (1964). Multiple comparisons using rank sums. *Technometrics*. **6**, 241-252.

Fernandez J.A., Wofford D.S., Horton J.L. (1985). Augmentation of wheat common root rot by *Fusarium acuminatum*. *Mycopathologia*. **90**, 177-179.

Figueroa-Castro D.M., Holtsford T.P. (2009). Post-pollination mechanisms in *Nicotiana longiflora* and *N. plumbaginifolia*: pollen tube growth rate, offspring paternity and hybridization. *Sexual Plant Reproduction*. **22**, 187-196.

Franich R.A., Gadgil P.D., Shain L. (1983). Fungistatic effects of Pinus radiata needle epicuticular fatty and resin acids on *Dothistroma pini*. *Physiological Plant Pathology*. **23**, 183-195.

Freire M.D.M., Gomes V.M., Corsini R.E., Machado O.L.T., De Simone S.G., Novello J.C., Marangoni S., Macedo M.L.R. (2002). Isolation and partial characterization of a novel lectin from *Talisia esculenta* seeds that interferes with fungal growth. *Plant Physiology and Biochemistry*. **40**, 61-68.

Fuerst E.P., Anderson J.V., Kennedy A.C., Gallagher R.S. (2011). Induction of polyphenol oxidase activity in dormant wild oat (*Avena fatua*) seeds and caryopses: A defense response to seed decay fungi. *Weed Science*. **59**, 137-144.

Fujii S., Kubo K., Takayama S. (2016). Non-self- and self-recognition models in plant self-incompatibility. *Nature Plants*. **2**, 16130.

Galen C., Stanton M.L. (1989). Bumble bee pollination and floral morphology: factors influencing pollen dispersal in the alpine sky pilot, *Polemonium viscosum* (Polemoniaceae). *American Journal of Botany*. **76**, 419-426.

Gardes M., Bruns T.D. (1993). ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Molecular Ecology*. **2**, 113-118.

Gardes M., White T.J., Fortin J.A., Bruns T.D., Taylor J.W. (1991). Identification of indigenous and introduced symbiotic fungi in ectomycorrhizae by amplification of nuclear and mitochondrial ribosomal DNA. *Canadian Journal of Botany*. **69**, 180-190.

Glawe G.A., Zavala J.A., Kessler A., Van Dam N.M., Baldwin I.T. (2003). Ecological costs and benefits correlated with trypsin protease inhibitor production in *Nicotiana attenuata*. *Ecology*. **84**, 79-90.

Goel A., Sheoran I.S. (2003). Lipid peroxidation and peroxide-scavenging enzymes in cotton seeds under natural ageing. *Biologia Plantarum*. **46**, 429-434.

Gowland K.M., Mathesius U., Clements M., Nicotra A.D. (2007). Understanding the distribution of three species of epiphytic orchids in temperate Australian rainforest by investigation of their host and fungal associates. *Lankesteriana*. **7**, 44-46.

Grant V. (1995). Sexual selection in plants: pros and cons. *Proceedings of the National Academy of Sciences of the USA*. **92**, 1247-1250.

Groten K., Nawaz A., Nguyen N.H.T., Santhanam R., Baldwin I.T. (2015). Silencing a key gene of the common symbiosis pathway in *Nicotiana attenuata* specifically impairs arbuscular mycorrhizal infection without influencing the root-associated microbiome or plant growth. *Plant Cell and Environment*. **38**, 2398-2416.

Hadar Y., Harman G.E., Taylor A.G. (1984). Evaluation of *Trichoderma koningii* and *T. harzianum* from New York soils for biological control of seed rot caused by *Pythium* spp. *Phytopathology*. **74**, 106-110.

Hadar Y., Harman G.E., Taylor A.G., Norton J.M. (1983). Reduction of *Pythium* seed rot by germination prior to planting and by *Enterobacter cloacae*. *Phytoparasitica*. **11**, 220.

Harman G.E. (1983). Mechanisms of seed infection and pathogenesis. *Phytopathology*. **73**, 326-329.

Harman G.E. (2000). Myths and dogmas of biocontrol. Changes in perceptions derived from research on *Trichoderma harzianum* T-22. *Plant Disease*. **84**, 377-393.

Harman G.E., Howell C.R., Viterbo A., Chet I., Lorito M. (2004). *Trichoderma* species - opportunistic, avirulent plant symbionts. *Nature Reviews Microbiology*. **2**, 43-56.

Harman G.E., Obregón M.A., Samuels G.J., Lorito M. (2010). Changing models for commercialization and implementation of biocontrol in the developing and the developed world. *Plant Disease*. **94**, 928-939.

Herrero M., Dickinson H.G. (1981). Pollen tube development in *Petunia hybrida* following compatible and incompatible intraspecific matings. *Journal of Cell Science*. **47**, 365-383.

Heslop-Harrison Y. (2000). Control gates and micro-ecology: the pollen-stigma interaction in perspective. *Annals of Botany*. **85**, 5-13.

Higashiyama T. (2010). Peptide signaling in pollen-pistil interactions. *Plant and Cell Physiology*. **51**, 177-189.

Hill J.P., Fernandez J.A., McShane M.S. (1983). Fungi associated with common root rot of winter wheat in Colorado and Wyoming. *Plant Disease*. **67**, 795-797.

Hiscock S.J., Allen A.M. (2008). Diverse cell signalling pathways regulate pollen-stigma interactions: The search for consensus. *New Phytologist*. **179**, 286-317.

Howell C.R. (1998). The role of antibiosis in biocontrol. In: Harman G.E., Kubicek C.P., eds. Trichoderma *and* Gliocladium*. Enzymes, biological control and commercial application*, Vol. 2. London, Taylor and Francis Ltd., pp. 173-183.

Howell C.R. (2003). Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. *Plant Disease*. **87**, 4-10.

Howell C.R. (2006). Understanding the mechanisms employed by *Trichoderma virens* to effect biological control of cotton diseases. *Phytopathology*. **96**, 178-180.

Hubbard J.P., Harman G.E., Hadar Y. (1983). Effect of soilborne *Pseudomonas* spp. on the biological control agent, *Trichoderma hamatum*, on pea seeds. *Phytopathology*. **73**, 655-659.

Hunter J. (1780). Account of an extraordinary pheasant. *Philosophical Transactions of the Royal Society of London*. **70**, 527-535.

Hunter J. (1837). An account of an extraordinary pheasant. In: Owen R., ed. *Observations on certain parts of the animal economy*. London, Longman.

Isshiki A., Akimitsu K., Yamamoto M., Yamamoto H. (2001). Endopolygalacturonase is essential for citrus black rot caused by *Alternaria citri* but not brown spot caused by *Alternaria alternata*. *Molecular Plant-Microbe Interactions*. **14**, 749-757.

Jolivet C., Bernasconi G. (2007). Within/between population crosses reveal genetic basis for siring success in *Silene latifolia* (Caryophyllaceae). *Journal of Evolutionary Biology*. **20**, 1361-1374.

Jones A.G., Ratterman N.L. (2009). Mate choice and sexual selection: What have we learned since Darwin? *Proceedings of the National Academy of Sciences of the USA*. **106**, 10001-10008.

Jones D.F. (1922). Selective fertilization and the rate of pollen tube growth. *Biological Bulletin*. **43**, 167-174.

Kärkkäinen K., Savolainen O., Koski V. (1999). Why do plants abort so many developing seeds: bad offspring or bad maternal genotypes? *Evolutionary Ecology*. **13**, 305-317.

Kato T., Yamaguchi Y., Namai T., Hirukawa T. (1993). Oxygenated fatty acids with anti-rice blast fungus activity in rice plants. *Bioscience Biotechnology and Biochemistry*. **57**, 283-287.

Kato T., Yamaguchi Y., Uyehara T., Yokoyama T., Namai T., Yamanaka S. (1983). Self defensive substances in rice plant against rice blast disease. *Tetrahedron Letters*. **24**, 4715-4718.

Kessler D., Bhattacharya S., Diezel C., Rothe E., Gase K., Schöttner M., Baldwin I.T. (2012). Unpredictability of nectar nicotine promotes outcrossing by hummingbirds in *Nicotiana attenuata*. *Plant Journal*. **71**, 529-538.

Kessler D., Gase K., Baldwin I.T. (2008). Field experiments with transformed plants reveal the sense of floral scents. *Science*. **321**, 1200-1202.

Kessler S.A., Shimosato-Asano H., Keinath N.F., Wuest S.E., Ingram G., Panstruga R., Grossniklaus U. (2010). Conserved molecular components for pollen tube reception and fungal invasion. *Science*. **330**, 968-971.

Kirkpatrick B.L., Bazzaz F.A. (1979). Influence of certain fungi on seed germination and seedling survival of four colonizing annuals. *Journal of Applied Ecology*. **16**, 515-527.

Kremer R.J. (1987). Identity and properties of bacteria inhabiting seeds of selected broadleaf weed species. *Microbial Ecology*. **14**, 29-37.

Kremer R.J., Begonia M.F.T., Stanley L., Lanham E.T. (1990). Characterization of rhizobacteria associated with weed seedlings. *Applied and Environmental Microbiology*. **56**, 1649-1655.

Kremer R.J., Schulte L.K. (1989). Influence of chemical treatment and *Fusarium oxysporum* on velvetleaf (*Abutilon theophrasti*). *Weed Technology*. **3**, 369-374.

Krügel T., Lim M., Gase K., Halitschke R., Baldwin I.T. (2002). *Agrobacterium*-mediated transformation of *Nicotiana attenuata*, a model ecological expression system. *Chemoecology*. **12**, 177-183.

Kuhl C., Tautenhahn R., Böttcher C., Larson T.R., Neumann S. (2012). CAMERA: an integrated strategy for compound spectra extraction and annotation of liquid hromatography/mass spectrometry data sets. *Analytical Chemistry*. **84**, 283-289.

Kumar D., Maurya N., Bharati Y.K., Kumar A., Kumar K., Srivastava K., Chand G., Kushwaha C., Singh S.K.*, et al.* (2014). *Alternaria* blight of oilseed *brassicas*: a comprehensive review. *African Journal of Microbiology Research*. **8**, 2816-2829.

Lankinen Å., Green K.K. (2015). Using theories of sexual selection and sexual conflict to improve our understanding of plant ecology and evolution. *Aob Plants*. **7**.

Lankinen Å., Skogsmyr I. (2002). Pollen competitive ability: the effect of proportion in two-donor crosses. *Evolutionary Ecology Research*. **4**, 687-700.

Lee T.D., Bazzaz F.A. (1986). Maternal regulation of fecundity: non-random ovule abortion in *Cassia fasciculata* Michx. *Oecologia*. **68**, 459-465.

Luu V.T., Weinhold A., Ullah C., Dressel S., Schoettner M., Gase K., Gaquerel E., Xu S., Baldwin I.T. (2017). *O*-acyl sugars protect a wild tobacco from both native fungal pathogens and a specialist herbivore. *Plant Physiology*. **174**, 370-386.

Lyons J.I., Newell S.Y., Buchan A., Moran M.A. (2003). Diversity of ascomycete laccase gene sequences in a southeastern U.S. salt marsh. *Microbial Ecology*. **45**, 270-281.

Mao W., Carroll R.B., Whittington D.P. (1998). Association of *Phoma terrestris*, *Pythium irregulare*, and *Fusarium acuminatum* in causing red root rot of corn. *Plant Disease*. **82**, 337-342.

Marshall D.L. (1988). Postpollination effects on seed paternity: mechanisms in addition to microgametophyte competition operate in wild radish. *Evolution*. **42**, 1256-1266.

Marshall D.L., Diggle P.K. (2001). Mechanisms of differential pollen donor performance in wild radish, *Raphanus sativus* (Brassicaceae). *American Journal of Botany*. **88**, 242-257.

Marshall D.L., Ellstrand N.C. (1986). Sexual selection in *Raphanus sativus*: experimental data on nonrandom fertilization, maternal choice, and consequences of multiple paternity. *American Naturalist*. **127**, 446-461.

Marshall D.L., Folsom M.W. (1991). Mate choice in plants: an anatomical to population perspective. *Annual Review of Ecology and Systematics*. **22**, 37-63.

Marshall D.L., Folsom M.W., Hatfield C., Bennett T. (1996). Does interference competition among pollen grains occur in wild radish? *Evolution*. **50**, 1842-1848.

Marshall D.L., Whittaker K.L. (1989). Effects of pollen donor identity on offspring quality in wild radish, *Raphanus sativus*. *American Journal of Botany*. **76**, 1081-1088.

Martínez Á.T., Speranza M., Ruiz-Dueñas F.J., Ferreira P., Camarero S., Guillén F., Martínez M.J., Gutiérrez A., del Río J.C. (2005). Biodegradation of lignocellulosics: microbial chemical, and enzymatic aspects of the fungal attack of lignin. *International Microbiology*. **8**, 195-204.

Mazer S.J., Moghaddasi A., Bello A.K., Hove A.A. (2016). Winning in style: Longer styles receive more pollen, but style length does not affect pollen attrition in wild *Clarkia* populations. *American Journal of Botany*. **103**, 408-422.

Mazer S.J., Snow A.A., Stanton M.L. (1986). Fertilization dynamics and parental effects upon fruit development in *Raphanus raphanistrum*: consequences for seed size variation. *American Journal of Botany*. **73**, 500-511.

McDonald M.B. (1999). Seed deterioration: physiology, repair and assessment. *Seed Science and Technology*. **27**, 177-237.

McKenzie C.L., Puterka G.J. (2004). Effect of sucrose octanoate on survival of nymphal and adult *Diaphorina citri* (Homoptera : Psyllidae). *Journal of Economic Entomology*. **97**, 970-975.

Melser C., Klinkhamer P.G.L. (2001). Selective seed abortion increases offspring survival in *Cynoglossum officinale* (Boraginaceae). *American Journal of Botany*. **88**, 1033-1040.

Mergoum M., Hill J.P., Quick J.S. (1998). Evaluation of resistance of winter wheat to *Fusarium acuminatum* by inoculation of seedling roots with single, germinated macroconidia. *Plant Disease*. **82**, 300-302.

Montalvo A.M. (1992). Relative success of self and outcross pollen comparing mixed- and single-donor pollinations in *Aquilegia caerulea*. *Evolution*. **46**, 1181-1198.

Moore J.C., Pannell J.R. (2011). Sexual selection in plants. *Current Biology*. **21**, R176-R182.

Morgan J.W. (1995). Ecological studies of the endangered *Rutidosis leptorrhynchoides*. I. Seed production, soil seed bank dynamics, population density and their effects on recruitment. *Australian Journal of Botany*. **43**, 1-11.

Mori T., Kuroiwa H., Higashiyama T., Kuroiwa T. (2006). Generative Cell Specific 1 is essential for angiosperm fertilization. *Nature Cell Biology*. **8**, 64-71.

Mummey D.L., Stahl P.D. (2004). Analysis of soil whole- and inner-microaggregate bacterial communities. *Microbial Ecology*. **48**, 41-50.

Nakamura R.R., Stanton M.L. (1989). Embryo growth and seed size in *Raphanus sativus*: maternal and paternal effects *in vivo* and *in vitro*. *Evolution*. **43**, 1435-1443.

Namai T., Kato T., Yamaguchi Y., Hirukawa T. (1993). Anti-rice blast activity and resistance induction of C-18 oxygenated fatty acids. *Bioscience Biotechnology and Biochemistry*. **57**, 611-613.

Ndakidemi P.A., Dakora F.D. (2003). Legume seed flavonoids and nitrogenous metabolites as signals and protectants in early seedling development. *Functional Plant Biology*. **30**, 729-745.

Nemeth M.B., Smith-Huerta N.L. (2002). Effects of pollen load composition and deposition pattern on pollen performance in *Clarkia unguiculata* (Onagraceae). *International Journal of Plant Sciences*. **163**, 795-802.

Ngugi H.K., Scherm H. (2004). Pollen mimicry during infection of blueberry flowers by conidia of *Monilinia vaccinii-corymbosi*. *Physiological and Molecular Plant Pathology*. **64**, 113-123.

Ngugi H.K., Scherm H. (2006). Biology of flower-infecting fungi. *Annual Review of Phytopathology*. **44**, 261-282.

Nunan N., Wu K., Young I.M., Crawford J.W., Ritz K. (2002). *In situ* spatial patterns of soil bacterial populations, mapped at multiple scales, in an arable soil. *Microbial Ecology*. **44**, 296-305.

O'Connell L.M., Johnston M.O. (1998). Male and female pollination success in a deceptive orchid, a selection study. *Ecology*. **79**, 1246-1260.

O'Donnell K. (1993). *Fusarium* and its near relatives. In: Reynolds D.R., Taylor J.W., eds. *The fungal holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematics*. Wallingford, UK: CAB International, pp. 225-233.

Ogle D.H. (2017). FSA: Fisheries Stock Analysis. R package version 0.8.12.

Ottaviano E., Mulcahy D.L. (1989). Genetics of angiosperm pollen. *Advances in Genetics Incorporating Molecular Genetic Medicine*. **26**, 1-64.

Papavizas G.C. (1985). *Trichoderma* and *Gliocladium*: biology, ecology, and potential for biocontrol. *Annual Review of Phytopathology*. **23**, 23-54.

Parker G.A. (1970). Sperm competition and its evolutionary consequences in the insects. *Biological Reviews*. **45**, 525-567.

Pasonen H.L., Käpylä M. (1998). Pollen-pollen interactions in *Betula pendula in vitro*. *New Phytologist*. **138**, 481-487.

Pasonen H.L., Pulkkinen P., Kapyla M. (2001). Do pollen donors with fastest-growing pollen tubes sire the best offspring in an anemophilous tree, *Betula pendula* (Betulaceae)? *American Journal of Botany*. **88**, 854-860.

Pasonen H.L., Pulkkinen P., Kapyla M., Blom A. (1999). Pollen-tube growth rate and seed-siring success among *Betula pendula* clones. *New Phytologist*. **143**, 243-251.

Pasonen H.L., Pulkkinen P., Kärkkäinen K. (2002). Genotype-environment interactions in pollen competitive ability in an anemophilous tree, *Betula pendula* Roth. *Theoretical and Applied Genetics*. **105**, 465-473.

Pelabon C., Albertsen E., Falahati-Anbaran M., Wright J., Armbruster W.S. (2015). Does multiple paternity affect seed mass in angiosperms? An experimental test in *Dalechampia scandens*. *Journal of Evolutionary Biology*. **28**, 1719-1733.

Pereira A.M., Lopes A.L., Coimbra S. (2016). Arabinogalactan proteins as interactors along the crosstalk between the pollen tube and the female tissues. *Frontiers in Plant Science*. **7**.

Pires N.D., Bemer M., Müller L.M., Baroux C., Spillane C., Grossniklaus U. (2016). Quantitative genetics identifies cryptic genetic variation involved in the paternal regulation of seed development. *Plos Genetics*. **12**.

Pitty A., Staniforth D.W., Tiffany L.H. (1987). Fungi associated with caryopses of *Setaria* species from field-harvested seeds and from soil under two tillage systems. *Weed Science*. **35**, 319-323.

Plitmann U. (1993). Pollen tube attrition as related to breeding systems in Brassicaceae. *Plant Systematics and Evolution*. **188**, 65-72.

Preston C.A., Baldwin I.T. (1999). Positive and negative signals regulate germination in the post-fire annual, *Nicotiana attenuata*. *Ecology*. **80**, 481-494.

Preston C.A., Betts H., Baldwin I.T. (2002). Methyl jasmonate as an allelopathic agent: Sagebrush inhibits germination of a neighboring tobacco, *Nicotiana attenuata*. *Journal of Chemical Ecology*. **28**, 2343-2369.

Qin Y., Leydon A.R., Manziello A., Pandey R., Mount D., Denic S., Vasic B., Johnson M.A., Palanivelu R. (2009). Penetration of the stigma and style elicits a novel transcriptome in pollen tubes, pointing to genes critical for growth in a pistil. *Plos Genetics*. **5**.

Queller D.C. (1983). Sexual selection in a hermaphroditic plant. *Nature*. **305**, 706-707.

RCoreTeam. (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org.

Read S.M., Clarke A.E., Bacic A. (1993a). Requirements for division of the generative nucleus in cultured pollen tubes of *Nicotiana*. *Protoplasma*. **174**, 101-115.

Read S.M., Clarke A.E., Bacic A. (1993b). Stimulation of growth of cultured *Nicotiana tabacum* W 38 pollen tubes by poly (ethylene glycol) and $Cu_{(II)}$ salts. *Protoplasma*. **177**, 1-14.

Riedel T., Groten K., Baldwin I.T. (2008). Symbiosis between *Nicotiana attenuata* and *Glomus intraradices*: ethylene plays a role, jasmonic acid does not. *Plant Cell and Environment*. **31**, 1203-1213.

Rigney L.P. (1995). Postfertilization causes of differential success of pollen donors in *Erythronium grandiflorum* (Liliaceae): nonrandom ovule abortion. *American Journal of Botany*. **82**, 578-584.

Rocha O.J., Stephenson A.G. (1991). Effects of nonrandom seed abortion on progeny performance in *Phaseolus coccineus* L. *Evolution*. **45**, 1198-1208.

Rodriguez A.E., Tingey W.M., Mutschler M.A. (1993). Acylsugars of *Lycopersicon pennellii* deter settling and feeding of the green peach aphid (Homoptera, Aphididae). *Journal of Economic Entomology*. **86**, 34-39.

Rondon M.R., Goodman R.M., Handelsman J. (1999). The earth's bounty: assessing and accessing soil microbial diversity. *Trends in Biotechnology*. **17**, 403-409.

Rose T.L., Conceição A.D., Xavier-Filho J., Okorokov L.A., Fernandes K.V.S., Marty F., Marty-Mazars D., Carvalho A.O., Gomes V.M. (2006). Defense proteins from *Vigna unguiculata* seed exudates: characterization and inhibitory activity against *Fusarium oxysporum*. *Plant and Soil*. **286**, 181-191.

Rotem J. (1994). *The genus* Alternaria*: biology, epidemiology and pathogenicity*. St Paul, USA: American Phytopathological Society.

Saeed A.I., Sharov V., White J., Li J., Liang W., Bhagabati N., Braisted J., Klapa M., Currier T.*, et al.* (2003). TM4: a free, open-source system for microarray data management and analysis. *Biotechniques*. **34**, 374-378.

Santhanam R., Luu V.T., Weinhold A., Goldberg J., Oh Y., Baldwin I.T. (2015). Native root-associated bacteria rescue a plant from a sudden-wilt disease that emerged during continuous cropping. *Proceedings of the National Academy of Sciences of the USA*. **112**, E5013-E5020.

Sasu M.A., Seidl-Adams I., Wall K., Winsor J.A., Stephenson A.G. (2010). Floral transmission of *Erwinia tracheiphila* by cucumber beetles in a wild *Cucurbita pepo*. *Environmental Entomology*. **39**, 140-148.

Schafer M., Kotanen P.M. (2004). Impacts of naturally-occurring soil fungi on seeds of meadow plants. *Plant Ecology*. **175**, 19-35.

Schuck S., Weinhold A., Luu V.T., Baldwin I.T. (2014). Isolating fungal pathogens from a dynamic disease outbreak in a native plant population to establish plant-pathogen bioassays for the ecological model plant *Nicotiana attenuata*. *Plos One*. **9**.

Schuman M.C., Heinzel N., Gaquerel E., Svatoš A., Baldwin I.T. (2009). Polymorphism in jasmonate signaling partially accounts for the variety of volatiles produced by *Nicotiana attenuata* plants in a native population. *New Phytologist*. **183**, 1134-1148.

Scribailo R.W., Barrett S.C.H. (1991). Pollen-pistil interactions in tristylous *Pontederia sagittata* (Pontederiaceae) . II. Patterns of pollen tube growth. *American Journal of Botany*. **78**, 1662-1682.

Sessitsch A., Weilharter A., Gerzabek M.H., Kirchmann H., Kandeler E. (2001). Microbial population structures in soil particle size fractions of a long-term fertilizer field experiment. *Applied and Environmental Microbiology*. **67**, 4215-4224.

Shingler V. (2003). Integrated regulation in response to aromatic compounds: from signal sensing to attractive behaviour. *Environmental Microbiology*. **5**, 1226-1241.

Shoresh M., Harman G.E. (2008). The molecular basis of shoot responses of maize seedlings to *Trichoderma harzianum* T22 inoculation of the root: a proteomic approach. *Plant Physiology*. **147**, 2147-2163.

Shoresh M., Harman G.E., Mastouri F. (2010). Induced systemic resistance and plant responses to fungal biocontrol agents. *Annual Review of Phytopathology*. **48**, 21-43.

Siemens D.H., Johnson C.D., Ribardo K.J. (1992). Alternative seed defense mechanisms in congeneric plants. *Ecology*. **73**, 2152-2166.

Sime K.R., Baldwin I.T. (2003). Opportunistic out-crossing in *Nicotiana attenuata* (Solanaceae), a predominantly self-fertilizing native tobacco. *BMC Ecol*. **3**, 6.

Simmons E.G. (1992). *Alternaria* taxonomy: current status, viewpoint, challenge. In: Chelkowski J., Visconti A., eds. Alternaria *Biology, Plant Diseases and Metabolites*. Amsterdam, Netherlands: Elsevier Science Publishers.

Sivasithamparam K., Ghisalberti E.L. (1998). Secondary metabolism in *Trichoderma* and *Gliocladium*. In: Harman G.E., Kubicek C.P., eds. Trichoderma *and* Gliocladium, Vol. 1. London, Taylor and Francis Ltd., pp. 139-191.

Skogsmyr I., Lankinen Å. (1999). Selection on pollen competitive ability in relation to stochastic factors influencing pollen deposition. *Evolutionary Ecology Research*. **1**, 971-985.

Skogsmyr I., Lankinen Å. (2002). Sexual selection: an evolutionary force in plants. *Biological Reviews*. **77**, 537-562.

Smit E., Leeflang P., Glandorf B., van Elsas J.D., Wernars K. (1999). Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. *Applied and Environmental Microbiology*. **65**, 2614-2621.

Smit E., Leeflang P., Gommans S., van den Broek J., van Mil S., Wernars K. (2001). Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. *Applied and Environmental Microbiology*. **67**, 2284-2291.

Smith C.A., Want E.J., O'Maille G., Abagyan R., Siuzdak G. (2006). XCMS: Processing mass spectrometry data for metabolite profiling using Nonlinear peak alignment, matching, and identification. *Analytical Chemistry*. **78**, 779-787.

Snow A.A. (1986). Pollination dynamics in *Epilobium canum* (Onagraceae): Consequences for gametophytic selection. *American Journal of Botany*. **73**, 139-151.

Snow A.A., Spira T.P. (1991). Pollen vigor and the potential for sexual selection in plants. *Nature*. **352**, 796-797.

Stanton M.A., Preßler J., Paetz C., Boland W., Svatoš A., Baldwin I.T. (2016). Plant-mediated pheromone emission by a hemipteran seed feeder increases the apparency of an unreliable but rewarding host. *New Phytologist*. **211**, 113-125.

Stanton M.L. (1994). Male-male competition during pollination in plant populations. *American Naturalist*. **144**, S40-S68.

Stephenson A.G., Travers S.E., Mena-Ali J.I., Winsor J.A. (2003). Pollen performance before and during the autotrophic-heterotrophic transition of pollen tube growth. *Philosophical Transactions of the Royal Society of London, B, Biological Sciences*. **358**, 1009-1018.

Stephenson A.G., Winsor J.A. (1986). *Lotus corniculatus* regulates offspring quality through selective fruit abortion. *Evolution*. **40**, 453-458.

Susko D.J., Lovett-Doust L. (1999). Effects of resource availability, and fruit and ovule position on components of fecundity in *Alliaria petiolata* (Brassicaceae). *New Phytologist*. **144**, 295-306.

Swanson R.J., Hammond A.T., Carlson A.L., Gong H., Donovan T.K. (2016). Pollen performance traits reveal prezygotic nonrandom mating and interference competition in *Arabidopsis thaliana*. *American Journal of Botany*. **103**, 498-513.

Takayama S., Isogai A. (2005). Self-incompatibility in plants. *Annual Review of Plant Biology*. **56**, 467-489.

Tautenhahn R., Böttcher C., Neumann S. (2008). Highly sensitive feature detection for high resolution LC/MS. *Bmc Bioinformatics*. **9**.

Taylor D.R., Saur M.J., Adams E. (1999). Pollen performance and sex-ratio evolution in a dioecious plant. *Evolution*. **53**, 1028-1036.

Templ M., Alfons A., Kowarik A., Prantner B. (2013). VIM: Visualization and imputation of missing values. R package version 4.0.0. http://CRAN.R-project.org/package=VIM.

Terras F.R.G., Eggermont K., Kovaleva V., Raikhel N.V., Osborn R.W., Kester A., Rees S.B., Torrekens S., Van Leuven F., *et al.* (1995). Small cysteine-rich antifungal proteins from radish: their role in host defense. *Plant Cell*. **7**, 573-588.

Terras F.R.G., Schoofs H.M.E., De Bolle M.F.C., Vanleuven F., Rees S.B., Vanderleyden J., Cammue B.P.A., Broekaert W.F. (1992). Analysis of two novel classes of plant antifungal proteins from radish (*Raphanus sativus* L.) seeds. *Journal of Biological Chemistry*. **267**, 15301-15309.

Terskikh V.V., Feurtado J.A., Borchardt S., Giblin M., Abrams S.R., Kermode A.R. (2005). *In vivo* ^{13}C NMR metabolite profiling: potential for understanding and assessing conifer seed quality. *Journal of Experimental Botany*. **56**, 2253-2265.

Thomma B.P.H.J. (2003). *Alternaria* spp.: from general saprophyte to specific parasite. *Molecular Plant Pathology*. **4**, 225-236.

Thornhill R. (1983). Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. *American Naturalist*. **122**, 765-788.

Thornhill R. (1984). Alternative female choice tactics in the scorpionfly *Hylobittacus apicalis* (Mecoptera) and their implications. *American Zoologist*. **24**, 367-383.

Torsvik V., Goksøyr J., Daae F.L. (1990). High diversity in DNA of soil bacteria. *Applied and Environmental Microbiology*. **56**, 782-787.

Trail F., Köller W. (1993). Diversity of cutinases from plant pathogenic fungi: purification and characterization of two cutinases from *Alternaria brassicicola*. *Physiological and Molecular Plant Pathology*. **42**, 205-220.

Varis S., Reininharju J., Santanen A., Ranta H., Pulkkinen P. (2010). Interactions during *in vitro* germination of Scots pine pollen. *Trees*. **24**, 99-104.

Vinale F., Sivasithamparam K., Ghisalberti E.L., Marra R., Woo S.L., Lorito M. (2008). *Trichoderma*-plant-pathogen interactions. *Soil Biology & Biochemistry*. **40**, 1-10.

von Dahl C.C., Winz R.A., Halitschke R., Kuhnemann F., Gase K., Baldwin I.T. (2007). Tuning the herbivore-induced ethylene burst: the role of transcript accumulation and ethylene perception in *Nicotiana attenuata*. *Plant Journal*. **51**, 293-307.

Walters C. (1998). Understanding the mechanisms and kinetics of seed aging. *Seed Science Research*. **8**, 223-244.

Wang H., Wu H.M., Cheung A.Y. (1996). Pollination induces mRNA poly (A) tail-shortening and cell deterioration in flower transmitting tissue. *Plant Journal*. **9**, 715-727.

Ward D.M., Weller R., Bateson M.M. (1990). 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature*. **345**, 63-65.

Warren H.L., Kommedahl T. (1973). Prevalence and pathogenicity to corn of *Fusarium* species from corn roots, rhizosphere, residues, and soil. *Phytopathology*. **63**, 1288-1290.

Waser N.M., Price M.V. (1989). Optimal outcrossing in *Ipomopsis aggregata*: seed set and offspring fitness. *Evolution*. **43**, 1097-1109.

Weindling R. (1932). *Trichoderma lignorum* as a parasite of other soil fungi. *Phytopathology*. **22**, 837-845.

Weinhold A., Baldwin I.T. (2011). Trichome-derived *O*-acyl sugars are a first meal for caterpillars that tags them for predation. *Proceedings of the National Academy of Sciences of the USA*. **108**, 7855-7859.

Westerman P.R., Liebman M., Heggenstaller A.H., Forcella F. (2006). Integrating measurements of seed availability and removal to estimate weed seed losses due to predation. *Weed Science*. **54**, 566-574.

Westoby M., Rice B. (1982). Evolution of the seed plants and inclusive fitness of plant tissues. *Evolution*. **36**, 713-724.

Weston P.H., Perkins A.J., Entwisle T.J. (2005). More than symbioses: orchid ecology, with examples from the Sydney Region. *Cunninghamia*. **9**, 1-15.

White T.J., Bruns T.D., Lee S.B., Taylor J.W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M.A., Gelfand D.H., Sninsky J.J., White T.J., eds. *PCR protocols: a guide to methods and applications*. San Diego, USA: Academic Press, pp. 315–322.

Wijaya R., Neumann G.M., Condron R., Hughes A.B., Polya G.M. (2000). Defense proteins from seed of *Cassia fistula* include a lipid transfer protein homologue and a protease inhibitory plant defensin. *Plant Science*. **159**, 243-255.

Wilkins K.A., Poulter N.S., Franklin-Tong V.E. (2014). Taking one for the team: self-recognition and cell suicide in pollen. *Journal of Experimental Botany*. **65**, 1331-1342.

Willson M.F. (1979). Sexual selection in plants. *American Naturalist*. **113**, 777-790.

Willson M.F. (1994). Sexual selection in plants: perspective and overview. *American Naturalist*. **144**, S13-S39.

Winsor J.A., Stephenson A.G. (1995). Demographics of pollen tube growth in *Cucurbita pepo*. *Canadian Journal of Botany*. **73**, 583-589.

Wright H.A., Neuenschwander L.F., Britton C.M. (1979). The role and use of fire in sagebrush-grass and pinyon-juniper plant communities. *U.S. Forest Service General Technical Report*. **INT-58**.

Wu J.Q., Kang J.H., Hettenhausen C., Baldwin I.T. (2007). Nonsense-mediated mRNA decay (NMD) silences the accumulation of aberrant trypsin proteinase inhibitor mRNA in *Nicotiana attenuata*. *Plant Journal*. **51**, 693-706.

Xia J.G., Psychogios N., Young N., Wishart D.S. (2009). MetaboAnalyst: a web server for metabolomic data analysis and interpretation. *Nucleic Acids Research*. **37**, W652-W660.

Xia J.G., Sinelnikov I.V., Han B., Wishart D.S. (2015). MetaboAnalyst 3.0-making metabolomics more meaningful. *Nucleic Acids Research*. **43**, W251-W257.

Yao C., Köller W. (1994). Diversity of cutinases from plant pathogenic fungi: cloning and characterization of a cutinase gene from *Alternaria brassicicola*. *Physiological and Molecular Plant Pathology*. **44**, 81-92.

Yao C.L., Köller W. (1995). Diversity of cutinases from plant pathogenic fungi: Different cutinases are expressed during saprophytic and pathogenic stages of *Alternaria brassicicola*. *Molecular Plant-Microbe Interactions*. **8**, 122-130.

Yedidia I., Benhamou N., Chet I. (1999). Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. *Applied and Environmental Microbiology*. **65**, 1061-1070.

Zelmer C.D., Cuthbertson L., Currah R.S. (1996). Fungi associated with terrestrial orchid mycorrhizas, seeds and protocorms. *Mycoscience*. **37**, 439-448.

Zettler L.W., Hofer C.J. (1998). Propagation of the little club-spur orchid (*Platanthera clavellata*) by symbiotic seed germination and its ecological implications. *Environmental and Experimental Botany*. **39**, 189-195.

Zhao P.F., Pan Q.F., Yu W.J., Zhao L.X. (2016). Dissect style response to pollination using metabolite profiling in self-compatible and self-incompatible tomato species. *Journal of Chromatography B, Analytical Technologies in the Biomedical and Life Sciences*. **1017**, 153-162.

Eigenständigkeitserklärung

Hiermit erkläre ich, dass mir die Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena bekannt ist. Die vorliegende Dissertation habe ich selbst angefertigt, keine Textabschnitte eines Dritten oder eigener Prüfungsarbeiten ohne Kennzeichnung übernommen und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen angegeben.

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