QUARTERLY REPORT

April 1, 2020 – June 30, 2020

Kentucky Tobacco Research & Development Center

College of Agriculture, Food and Environment

UNIVERSITY OF KENTUCKY
College of Agriculture, Food and Environment

MEMORANDUM

DATE: July 29, 2020

TO: Kentucky Tobacco Research Board Members
Legislative Research Commission

FROM: Dr. Ling Yuan
Managing Director, KTRDC

SUBJECT: Kentucky Tobacco Research & Development Center
Quarterly Report for April 1, 2020 – June 30, 2020

Enclosed is a copy of the Kentucky Tobacco Research & Development Center's Quarterly Report for April 1, 2020 – June 30, 2020.

If you have any questions, please feel welcome to contact me at (859) 257-5798 or email lyuan3@uky.edu.

Enc.

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EXECUTIVE SUMMARY

Introduction

The legislation (KRS 248.510 - 248.580) which provides funds in support of the research programs at the Kentucky Tobacco Research and Development Center (KTRDC) requires that a quarterly research report be submitted to the Kentucky Tobacco Research Board (KTRB) and the Legislative Research Commission.

The overall reporting plan is:

January 1	-	March 31:	Selected topics
April 1	-	June 30:	Selected topics
July 1	-	September 30:	Selected topics
October 1	-	December 31:	Annual comprehensive report

As required by KRS 248.570, a financial report covering expenditures for the relevant proportion of the April 1, 2020 – June 30, 2020 fiscal year is included in this report.

The news and research publications provided in this quarterly report are a representative selection of the Center's output. For a full description of all KTRDC research and activities please refer to the KTRDC Annual Report.

Quarterly News

- In June, it was decided that the TSRC (Tobacco Science Research Conference), due to be held in Boston, Massachusetts at the end of September, will be postponed to 2021, due to the COVID 19 pandemic. This conference is always well-attended by KTRDC scientists, many presenting papers and posters. For the last several years, this conference has also been the venue for well-attended annual workshops on the reference products.

- In June, it was decided that the CORESTA conference, due to be held in Vienna, Austria in mid-October, will be online, due to the COVID 19 pandemic. There are always presentations from KTRDC scientists, and KTRDC is very involved in the board, the Scientific Commission and a number of working groups. Several KTRDC scientists will be presenting papers and posters.

- KTRDC staff have continued to work through the pandemic, with strict precautions in place, as there are ongoing grants and many experiments which cannot be halted. Those who can work from home have been doing so, so the number of people in the building is greatly reduced. Social distancing is observed, face masks are mandatory and lab staff are carefully spread out, rotating their hours and locations.

- Throughout May and June, KTRDC scientists, in collaboration with UK agricultural engineers, worked on submitting a large grant. The grant application has now been submitted, but the results have not yet been announced.

- Transplanting at the farm was late, because of continual rain, and was done through the first few weeks of June. So far, the crop is very even.

- Twelve summit grants were awarded in May, for a total of $197,108. The requested emphasis of the grants was in three areas: (1) TSNA accumulation (2) low nicotine tobacco and (3) cigar tobacco. Proposals were received covering all of these areas, as well as two proposals on other topics.
    1. Further evaluation of leaf moisture and tobacco-specific nitrosamine effects of potassium chloride application to dark tobacco (*Andy Bailey*)
    2. Leaf chemistry of Pale Yellow Dark Burley (PYDB) (*Colin Fisher*)
    3. A transcriptomic approach to study potassium chloride-dependent suppression of tobacco-specific nitrosamines (*Sanjay Singh*)
    4. Reducing tobacco-specific nitrosamines in burley tobacco by weaponizing plant stress memory (*Patrick Perry*)
    5. The effect of low alkaloid (LA) burley varieties and nitrogen rate on nutrient assimilation, nitrogen use efficiency, yield and alkaloids (*Patrick Perry*)
    6. Engineering the long-distance root-to-shoot transport mechanism of nicotine to control its accumulation in the leaf (*Barun Patra*)
    7. Comparative study of aphid infestation in low- and moderate-nicotine tobacco in the presence / absence of CBT diols – greenhouse study (*Joe Zhou*)
    8. Comparative study of aphid infestation in low- and moderate-nicotine tobacco in the presence / absence of CBT diols – field study (*Toni Kroumova*)
    9. Cigar tobacco chemistry (*Colin Fisher*)
    10. Determination of carbonyls in cigar tobacco leaf and cigar products (*Huihua Ji*)
    11. Evaluation of novel biological control Rhizobacteria for management of angular leaf spot in dark tobacco (*Natalia Martinez*)
    12. Sequencing the nuclear genome of Nicotiana langsdorffii (*David Zaitlin*)
- The June board meeting was held via Zoom, because of the pandemic. The KTRDC Director, Ling Yuan, gave an update on the KTRDC activities and budget. Income for the current year was higher than the budgeted projected income.
- The proficiency testing (PT) program continues to be well patronized.
    - The CIG-2020A round opened in January. Due to the COVID-19 pandemic, dates were adjusted based on participant feedback. The final report for the round of PT was completed on August 30.
    - The CIG-2020B round opening was delayed until mid-April due to the pandemic and is currently underway with 23 kits sold to 19 unique institutions.
    - The CIG-2020C round opening was delayed until mid-June due to the pandemic and is currently underway with 13 kits sold to nine unique institutions. Additional participants are expected as the kits will be available for sale until the end of September.
    - The SMK-2020D is scheduled to open at the end of August and will be the first smokeless PT round that CTRP will offer following the A2LA scope expansion in

March of 2020. Because the smokeless PT round does not require smoking machines, it is anticipated that new unique institutions will participate in PT testing.

- The four certified reference smokeless tobacco products were to be used in a CORESTA collaborative study in 2020. Due to the COVID-19 pandemic, this study has been delayed until 2021.
- CTRP introduced four reference cigars for research purposes in October 2019. The 1C1 (large machine made cigar) has sold 2,000 units. The 1C2 (machine made little cigar) has sold 5,400 units. The 1C3 (cigarillos) has sold 4,200 units. The 1C4 (large machine made cigar with a natural wrapper) has sold 1,344 units. Work is underway to produce three certified reference cigar products as part of a cooperative agreement with the FDA.

The KTRDC Quarterly Reports include copies and brief summaries of research done by KTRDC scientists. I would like to thank Dr. Ruth McNees, Huihua Ji and Antoaneta Mihaylova Kroumova for their help with writing the summaries.

Summary of Selected Research Topics

Report #1 "The Microbiology of Hemp Retting in a Controlled Environment: Steering the Hemp Microbiome towards More Consistent Fiber Production." Audrey Law, Ruth McNees and Luke Moe

The process of generating natural fibers from hemp requires the material undergo retting in the field prior to processing. The retting process for hemp is similar to that for hay, where the stalks are cut, and the vegetative material is left in the field for a period of time, the exact duration of which can be difficult to predict. During this time, the microbial population degrades the cellulose material which holds the fibers together. In an effort to help determine the microbial impact for the duration of retting, a controlled experiment where three varieties of hemp were exposed to variable amounts of moisture to simulate dew in the field, heavy rain, and heavy rain resulting in soil splashing on the stalks. The microbial population was evaluated at six time points which covered fresh stalk, optimally-retted, and over-retted material. The results reveal that the bacterial population stays consistent throughout retting, and that Proteobacteria dominates all samples tested. Samples with greater moisture content achieved optimal-retting quicker than other samples and showed an increase in abundance of Bactreroidetes in samples that correspond to optimally-retted or over-retted samples. Furthermore, samples that simulated soil splashing had a similar bacterial population as that of the heavy-rain samples, indicating that the bacterial population at the time of harvest is resilient during the retting process.

Report #2 "Determination of tobacco alkaloid enantiomers using reversed phase UPLC/MS/MS." Huihua Ji, Ying Wu, Franklin Fannin and Lowell Bush

Alkaloids are the precursors of tobacco-specific N'-nitrosamines (TSNAs) which are carcinogenic compounds. The isomers of accumulated nicotine, nornicotine and NNN

impact their biological activities. Many works have been done to change the accumulation of nornicotine in tobacco in the recent past, including the screening of parental seed source plants and releasing of Low Converter varieties. However, though a great amount of effort and expense have been involved in producing ultra-low converter commercial varieties, it is possible that there will be no benefit in terms of reduced carcinogenicity as the different alkaloid enantiomers have different pharmacological activities. (S)-nicotine is more toxic than (R) -nicotine, while (S)-NNN (produced from (S)-nornicotine) is more carcinogenic than the (R)-NNN. An accurate measurement of the isomers of alkaloids will allow us to better understand the reducing toxicity of tobacco and tobacco products. In this study, a method for the determination of the isomers of alkaloids in tobacco was developed for routine analysis.

Report #3 "Patterns of inheritance of acylsugar acyl groups in selected interspecific hybrids of genus Nicotiana". Antoaneta Mihaylova Kroumova, Ivan Artiouchine, Victor D. Korenkov and George Wagner

Wild Nicotiana species are a great resource for genetic variability of the acyl sugars (ASs), produced on the plants surface by glandular trichomes. ASs with medium-chain length acyl groups are considered more potent as natural insecticides. If the medium-chain acids can be introduced into the ASs of tobacco crops, they might enhance pest and insect resistance. One way of introducing new secondary metabolites is via hybridization. The objective of this work was to analyze the acyl composition of acyl sugars from selected Nicotiana species and to follow the inheritance pattern in their hybrids. The following patterns were observed: a single parental resemblance (paternal in N. tabacum cv. Turkish Samsun × N. benthamiana, and maternal in N. tabacum cv. Samsun-nn × N. otophora), missing groups (in N. excelsiana), and appearance of novel groups (N. excelsior hybrids). Complementary inheritance was observed but the ratio of acyl groups and the major acyl groups did not resemble the profile of either parent (N. t. Samsun-nn × N. benthamiana, dark tobacco × N. otophora, and flue-cured tobacco × N. otophora). Selective inheritance of some acyl groups in the hybrids of N. benthamiana (4- and 5-methylheptanoic isomers) or N. alata (octanoate) was found. Suggestions were given to explain certain patterns of inheritance. The data presented in this paper contribute to the body of knowledge about the effect of interspecific hybridization on the secondary metabolites by including acylsugar acyl groups that have not been studied previously.

agronomy

MDPI

Article

The Microbiology of Hemp Retting in a Controlled Environment: Steering the Hemp Microbiome towards More Consistent Fiber Production

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Abstract: Industrial hemp (*Cannabis sativa* L.) production is increasing dramatically in the US due to recent changes which lift restrictions on the growth and sale of hemp products; however, due to the decades-long prohibition of hemp, there is a lack of current research with respect to varieties and best agricultural practices for the many uses of this versatile crop. Natural fiber production relies on retting, a microbially-mediated process necessary for the separation of fibers from the plant which can occur unevenly in the field environment and result in inconsistent fiber quality and lower processing efficiency. In this study, the microbiome of hemp stalks is investigated throughout the retting process using 16S rRNA gene amplicon sequencing using the Illumina MiSeq platform. Field retting conditions were simulated in a controlled greenhouse environment in order to determine the effects of different moisture levels and soil contact on the retting process. Samples were taken over six time points, reflecting the community of freshly cut stalks to optimally-retted material, and finally over-retted material showing degraded fibers. The results show a very consistent population throughout retting, dominated primarily by *Proteobacteria*, but showing an increase in the abundance of the *Bacteroidetes*, namely *Chryseobacterium*, in time points corresponding to optimally-retted and over-retted stalks in treatments receiving higher moisture levels, but not in the low-moisture treatment. Soil application did not appear to influence the microbial community throughout retting, indicating a resilient population present in and on the hemp stalks at harvest.

Keywords: hemp microbiome; fiber crops; fiber retting

1. Introduction

Hemp (*Cannabis sativa* L.) is one of the oldest crops continuously cultivated by humans, with hempen cloth found dating to over 6000 years ago [1]. In the United States, hemp was introduced in the mid- to late-18th century when hemp fibers were used to produce fabrics, twine, and paper [2], with Kentucky leading the US in production from the civil war era to WWII [3]. Hemp production would eventually decline due to decreased demand for hemp sails and ropes in modern ships, the rise in demand for fabrics made from cotton and synthetic fibers, and innovations in the wood pulping industry for making paper. Production of hemp was all but eliminated in North America after the passing of the US Marihuana Tax Act and Canadian Opium and Narcotics Act in 1938, which prohibited all cultivation of *Cannabis* without government permission [4]. Currently, as the result of increased demand for renewable and sustainable materials, hemp has experienced a renewed interest as a fiber crop around the world, with Australia planting the first crop in 1990, followed by England in 1993, Germany in 1995, Canada in 1998, and the US in 2014 [1].

Presently, synthetic fibers dominate the market when it comes to industrial applications [1]. Hemp is considered an attractive alternative to synthetics in part due to the versatility of the plant fibers—they can be used in textiles, yarns, paper, construction materials, auto parts, and composites [2]. The automotive industry has been especially influential for the production of industrial hemp due to the lighter weight and improved structural properties when compared to glass and resin-based materials [5]. Additionally, using hemp for paper production could reduce the need for wood pulping due to the decreased cost of pulping hemp and the fact that hemp can be recycled twice as many times as wood paper [5]. The projected profitability of fiber hemp is difficult to assess due to current limitations of processing facilities and uncertain demand in the manufacturing industry; however, estimates from various sources suggest fiber hemp could be comparable to other major field crops [3].

The 2014 US Farm Bill allowed state departments of agriculture to approve hemp pilot programs for farmers, colleges, and universities. The long lapse in hemp cultivation, however, has set the US behind the rest of the world in experience and research. For hemp fiber crops in particular, one of the main problems that has been identified by farmers and fiber processors concerns field retting, also known as dew retting, whereby hemp stalks are cut and left in the field for a period of time before baling. During retting, microbial activity degrades polysaccharides, mainly pectin, that bind the bast fibers to the hurd core such that they can be separated by a mechanical process called decortication [6]. This is distinct from the process of water retting, used mainly for textile quality fibers, in which the stalks are submerged in water which may contain additional enzymes and/or microbial cultures [6]. Field retting is the most common method used by western countries, as it is considerably cheaper and does not produce waste water. The fiber quality from optimally retted hemp is suitable for many industrial uses, though factors such as environmental conditions and grower inexperience can result in less valuable fiber grades and poor uniformity; fibers that are under-retted cannot be decorticated effectively and over-retted fibers are weaker and less valuable [6]. Processors who buy field retted hemp from farmers in order to make the kinds of fiber products that can be used in industry need a steady supply and consistent quality, however the economics of the industrial fiber market are, at this time, prohibitive to practices or inputs that increase costs.

Field retting is the most practical method of hemp fiber production for farmers in the US, but little research has been done on the microbiota associated with hemp stalks during field retting, or on applied practices that improve the quality and consistency of the fiber without substantially increasing cost. Work here describes the microbial communities associated with hemp in a controlled greenhouse retting study. Three varieties were chosen for the study, two common fiber varieties, Futura 75 and Felina 32, and SS Alpha, an experimental variety that is being tested for its suitability for fiber crops in the US. We hypothesize that the bacterial population of the stalk will shift over retting time, correlating with under-retted and optimally-retted, as well as considerably over-retted material, and that treatments manipulating moisture and access to environmental microbiota will influence the population at these time points. Information about how these factors affect the microbial population involved in the retting process and whether or not it differs by variety can lead to improvements in the quality and value of field-retted hemp, thereby increasing profitability for farmers and encouraging the inclusion of more natural fibers in industry and manufacturing.

2. Materials and Methods

Industrial hemp (*Cannabis sativa* L.) varieties Futura 75 (FU), Felina 32 (FE), and SS Alpha (SSa) were planted on 1 July 2016 at Spindletop Farm in Lexington, KY (38.125885, −84.497585) as part of a variety trial. Plots were tilled conventionally and fertilized with 150 lb/ac (168.3 kg/ha) of nitrogen in the form of granulated urea (incorporated pre-planting), and planted with a seed density of 40 lb/ac (44.83 kg/ha). Stalks were cut manually at the base of the plant on 28 September 2016. At this time, varieties FE and FU had flowered and were in the early stages of senescence, which is typically when hemp stalks are cut for field retting; however the SSa variety was still in vigorous vegetative growth and had not flowered. As an experimental variety being tested for suitability for fiber production in

Kentucky, it was discovered in trials that the SSa performed very differently than the other varieties tested, in that vigorous vegetative growth continued throughout the growing season without flowering, up to when the plants were killed by hard frost.

The cut stalks were prepared for retting in the greenhouse by measuring the total length of each stalk and cutting a 4 ft (73.44 cm) section from the center before placing in constructed retting boxes. The retting box consisted of three wooden frames (3 m × 1.5 m), separated into three equal sections (3.33 m), which were set on benches in the greenhouse and overlaid with 4 mil plastic, with 4 layers of burlap lining the bottom of each section. The burlap was intended to absorb and retain moisture from applied treatments as soil would in the field, and to prevent stalks getting uneven amounts of moisture from the pooling of water on the plastic lining. Each frame represented a treatment, and each section within the frame contained 21 stalks of an individual variety.

Three treatments were applied to each variety during greenhouse retting: low moisture (LM), high moisture (HM) and high moisture with soil slurry (SHM). The LM treatment consisted of 200 mL autoclaved deionized water misted over stalks every day using a surface sterilized hand-held sprayer. Additionally, 1 L of water was used to wet the burlap (avoiding stalks) every other day to simulate moisture retained in the soil in a field environment. The HM treatment consisted of 2 L of sterile deionized water showered over stalks from a sterile plastic watering can every two days, and misting with 200 mL sterile deionized water as above on days in between. The SHM treatment was the same as HM, except 1 g L^{-1} of soil collected from the field where the hemp was grown was added to the 2 L of water showered over stalks to simulate exposure to soil microorganisms during heavy rain. Six time points were used for sampling over the course of retting, corresponding to 24 h after placing stalks in the greenhouse, before any applied treatment (T1), and every seven days thereafter until it was determined the majority of stalks were sufficiently retted (bast fiber readily separated from the hurd core, but could not be easily broken by hand), which took between four and five weeks (T4-T5). Stalks were turned over in their boxes once during the study, at T3, as it is typical to turn hemp in the field during retting at least once. An additional sample was taken after seven weeks to examine highly over-retted conditions, in which significant fiber degradation was observed through easy breakage and visible fungal growth on the stalks and fibers (T6). Retting quality was ascertained by observation (color) and mechanical properties (ease with which bast and hurd separate by hand) to mimic what is done in a conventional agricultural field to determine when the majority of the crop is well-retted and ready to be baled for processing.

Three stalks were sampled from the each treatment at each time point. A six inch section was removed from the center of each stalk and cut into 1 cm pieces which were processed with a paddle blender (Bag Mixer 400, Interscience) using BagPage XR filter bags at high speed and closest setting for 5 min in 150 mL 100 mM sodium phosphate buffer, pH 7.0. Buffer filtered from the plant debris was poured into 250 mL sterile centrifuge bottles and centrifuged at 10,000× *g* for 20 min. The supernatant was discarded and the pellet resuspended in 2 mL of the phosphate buffer, then transferred to 2 mL microcentrifuge tubes and centrifuged again at 10,000× *g* for 5 min. The buffer was discarded and the pellet frozen at −20 °C. DNA extraction from frozen pellets and field soil samples used in SHM treatments was done using the MOBIO Power soil kit (Carlsbad, CA, USA).

DNA from hemp and soil samples was shipped to The University of Michigan Microbial Systems Molecular Biology Laboratory core sequencing facility (http://microbe.med.umich.edu/services/microbial-community-analysis) for PCR amplification and sequencing of the V4 region of the 16S rRNA gene on the Illumina Miseq platform (dual-barcoded, paired-end reads, 2 × 250 flow cell) according to Kozich et al. [7].

Sequence data from MiSeq analysis was processed using Mothur software (v1.40.5) following the MiSeq SOP (https://www.mothur.org/wiki/MiSeq_SOP, accessed July 2018) [7,8]. Briefly, paired-end reads were assembled into contigs, sequences were filtered for length, ambiguous bases, and homopolymer regions, then aligned to a SILVA reference alignment of the V4 region (SSU Silva 132). Pre-clustering to merge highly similar (2 bp or less mismatch) sequences was followed

by the removal of chimeras and 16S rRNA sequences derived from mitochondrial and chloroplast DNA. Operational taxonomic units (OTUs) were defined using a cutoff of 0.03 (97% similarity) and taxonomic classification was assigned based on Ribosomal Database Project (RDP) reference sequences (version 16, February 2016). The hemp data set was normalized to 2234 sequences per sample, which resulted in 2629 OTUs. This sampling depth resulted in at least 90% coverage using Good's Non-parametric Coverage estimator, with most samples greater than 97% [9,10]. Soil samples used in the SHM treatment were processed separately and normalized to 10,737 sequences per sample, resulting in 8661 OTUs. Two sampling depths were chosen for hemp and soil sequences because at 2234 sequences per sample, the soil samples were below 80% coverage. The soil and hemp samples were not statistically compared in this study, therefore different cutoffs for each of the data sets were chosen for maximum data retention. Raw sequence reads for all samples in this study were uploaded to the NCBI BioProject database under accession number PRJNA494847.

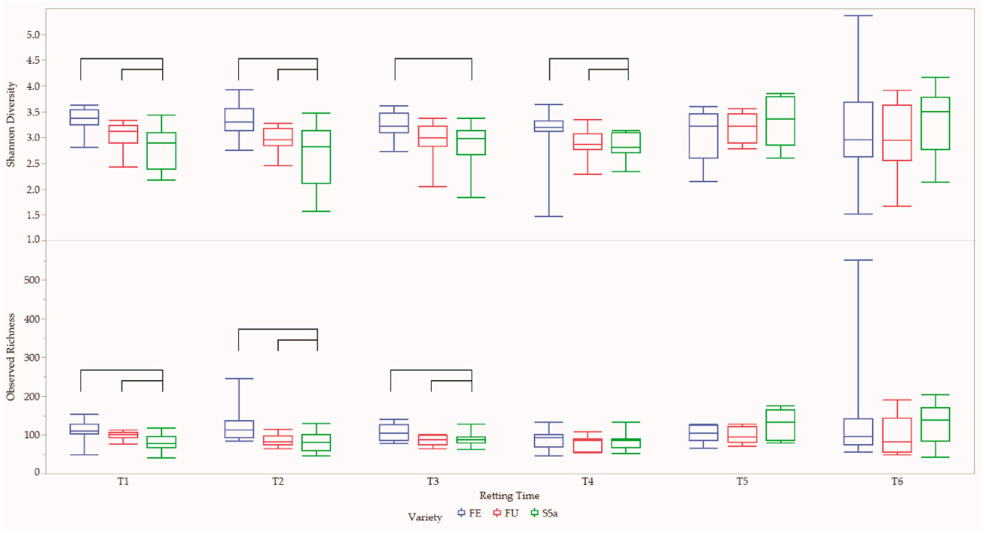
Statistical analysis was performed using programs in Mothur [8], Phyloseq package for R [11], and LEfSe (Linear discriminate analysis Effect Size) [12]. In Mothur, analysis of similarities (ANOSIM) [13] was used to compare bacterial community structure, and Indicator Species Analysis (ISA) [14] was used to identify individual OTUs whose presence was strongly indicated according to sample groups. The LEfSe program is a tool used to detect features that are most likely to explain differences between two or more sample groups. The Phyloseq program for R was used to calculate alpha diversity measures of Observed Richness and Shannon diversity. Jmp Pro software (version 13.2) was used to compare alpha diversity measures and abundance using the Kruskal-Wallis rank sum test, with significant results followed by Wilcoxon each-pair signed-rank test. The threshold used to determine statistical significance is $\alpha = 0.05$ unless otherwise indicated.

3. Results

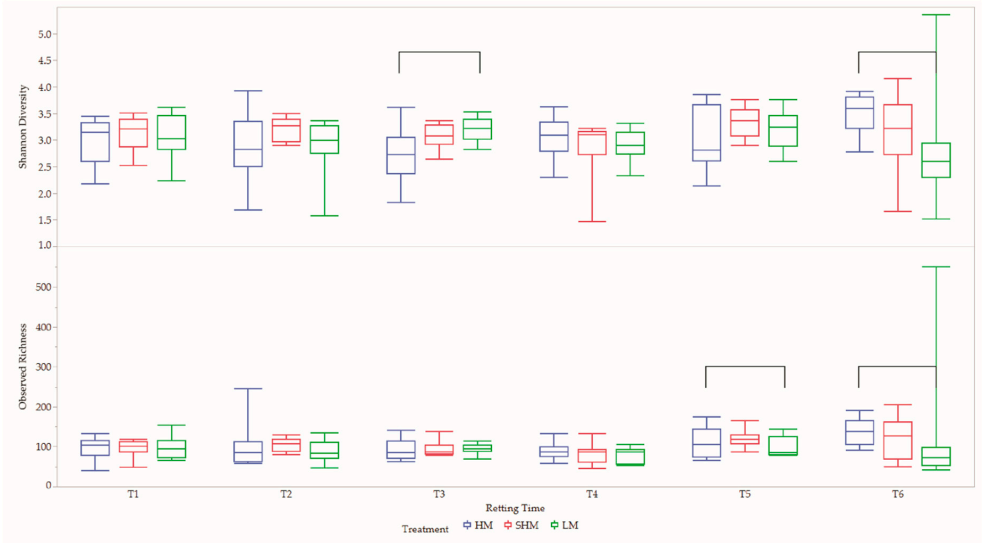
3.1. Diversity/Community Similarity

The three varieties (FE, FU, SSa) were compared at each time point. At T1, the SSa variety showed significantly lower alpha diversity values than FE and/or FU (Figure 1). Observed richness of variety SSa was lower than FE and FU for the first three time points, and showed lower Shannon diversity at T4, but generally, significant differences by variety decreased at each time point, with no further varietal differences after T4. ANOSIM comparisons between all varieties at T1-T2 were significant—at time points T3 and T4, SSa still showed significant differences from the other varieties, and by T4-T5 no significant differences between varieties remain (Table 1).

Treatments (LM, HM, SHM) within each variety (FE, FU, SSa) and among all varieties were compared over time and at each time point. Within each variety, all treatments compared at each time point and over time, showed no significant differences in alpha diversity measures and ANOSIM analysis (not shown). When treatments were compared at each time point including all varieties, no differences were seen for time points T1 and T2. At T3, the LM treatment was significantly lower than HM for measures Shannon diversity, and at T6 the LM treatment was lower for observed richness as well as Shannon diversity compared to the HM treatment (Figure 1b). ANOSIM global values were significant for T3, T5 and T6, with pair-wise comparisons showing significance between LM and HM treatments (Table 1), although R values were small (<0.3).



(**a**)



(**b**)

Figure 1. Alpha diversity (**a**) Observed richness and Shannon diversity by variety (**b**) Observed richness and Shannon diversity by treatment. Lines connecting box plots indicate significant difference using Wilcoxon each-pair signed-rank text, $\alpha = 0.05$.

Table 1. ANOSIM comparisons over time by variety and treatment [1].

	Retting Time					
	T1	**T2**	**T3**	**T4**	**T5**	**T6**
Variety	**0.337**	**0.263**	**0.228**	**0.228**	0.064	0.045
FE-FU	**0.220**	**0.206**	**0.152**	**0.152**		
FE-SSα	**0.486**	**0.317**	**0.308**	**0.308**		
FU-SSα	**0.376**	**0.302**	**0.235**	**0.235**		
Treatment	0.031	−0.017	**0.104**	0.031	**0.208**	**0.154**
D-HR			**0.220**		**0.353**	**0.345**
D-SS			0.098		**0.209**	0.088
HR-SS			0.000		0.021	0.015

[1] Global tests with *p*-values greater than 0.05 were followed by pair-wise comparisons. R-values in red bold indicate significance at $\alpha = 0.001$, R-values in black bold indicate significance at $\alpha = 0.05$.

3.2. Taxonomic Distribution and Community Profile

A total of 20 Phyla were present in the dataset; the most abundant were Proteobacteria (85.55%), Bacteroidetes, (7.58%), Bacteria_unclassified (2.78%), Actinobacteria (2.27%), and Firmicutes (1.13%), with Deinococcus-Thermus, Verrucomicrobia, Planctomycetes, Acidobacteria, candidate_division_WPS-1, Armatimonadetes, Candidatus_Saccharibacteria, Gemmatimonadetes, Fusobacteria, Chloroflexi, Tenericutes, Chlamydiae, SR1, candidate_division_WPS-2, and Nitrospirae making up the remaining 0.71%. Both HM and SHM treatments show a slight but significantly higher abundance in Bacteroidetes and a decrease in Proteobacteria at T5 and T6 (Figure 2). The Bacteroidetes phylum contained 319 OTUs including 40 genera; only one genus from the 20 most abundant OTUs (approximately 75% of the total abundance in the dataset) was a Bacteroidetes (Chryseobacterium), the others were comprised of 13 genera within Proteobacteria (Figure 3). While lower in abundance, and not statistically significant, several other OTUs in the Bacteroidetes phylum classified as Sphingobacteriaceae, Flavobacterium, Pedobacter, and Mucilaginibacter also increased in abundance from T4 to T6 (not shown).

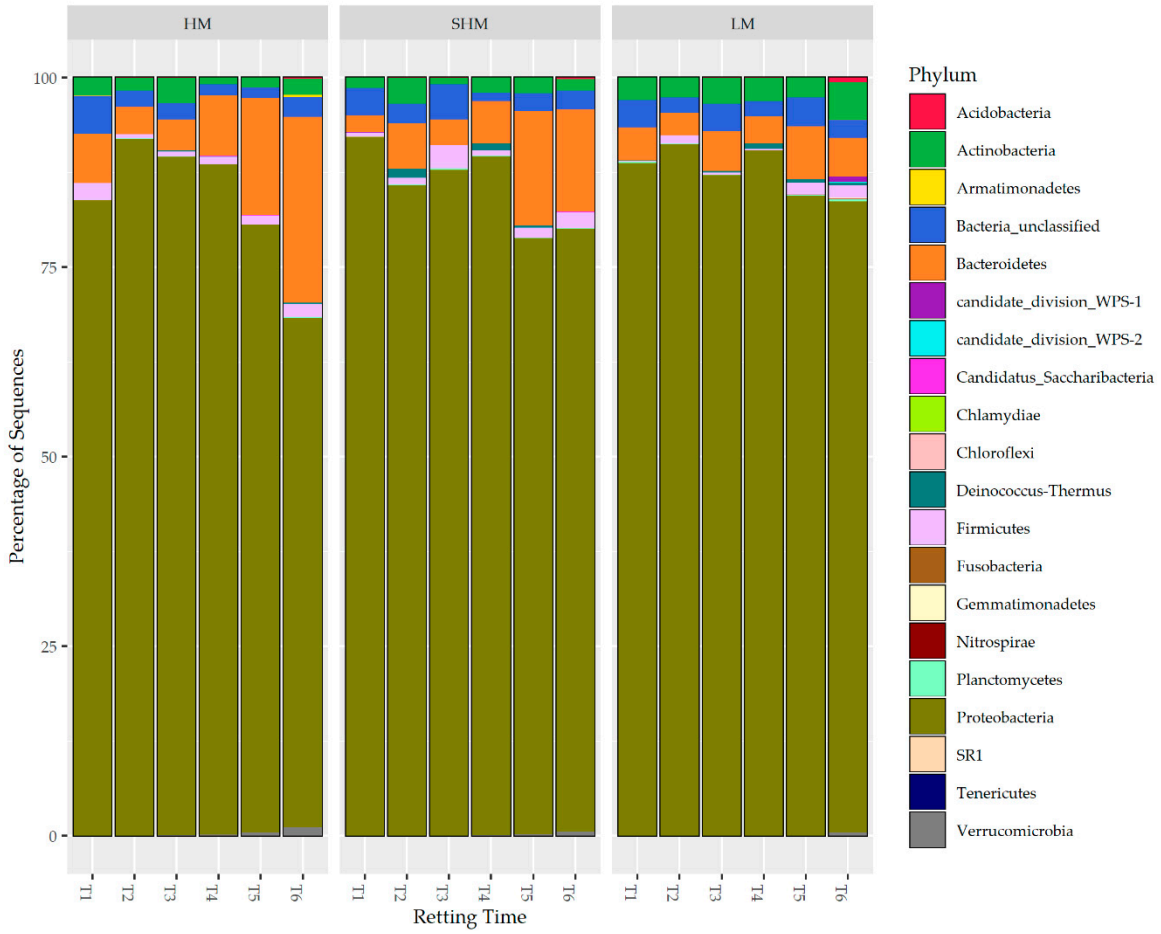


Figure 2. Relative abundance of Phyla according to treatment over time.

The SHM treatment was exposed to field soil, in the form of 1 g L^{-1} added to the water it received. We hypothesized that the field environment could contribute to the microbial profile by splashing onto stalks during rain and contact with the ground. While the soil slurry profile showed many of the same phylum groups as the hemp stalks, only three of the 20 most abundant genera were shared with hemp samples: *Massilia*, *Pseudomonas*, and *Sphingomonas*.

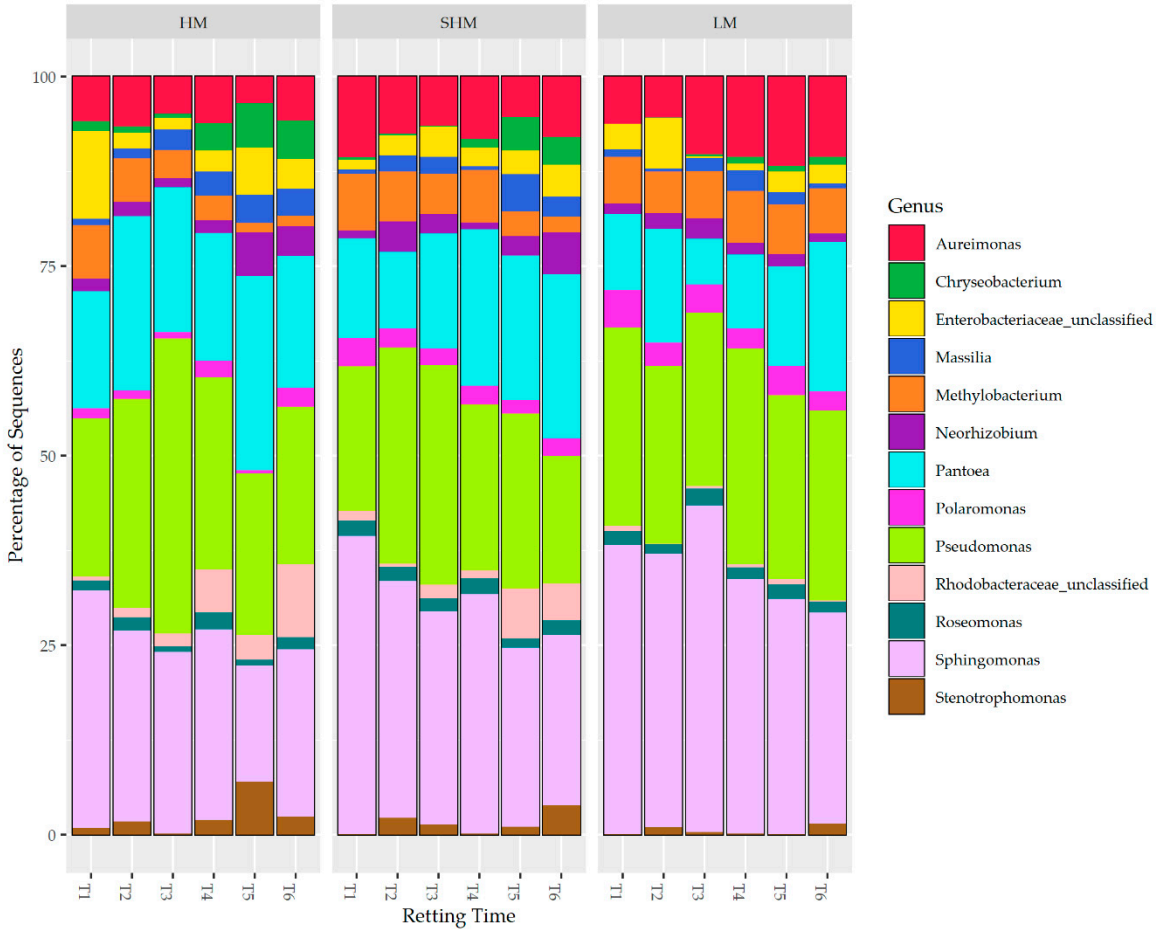


Figure 3. Relative abundance of genera composed of the 20 most abundant OTUs, according to treatment over time.

4. Discussion

Improving hemp fiber crop practices in order to make the resulting products more competitive for industrial use remains a goal that is largely unfulfilled at this time. This study, along with previous research, looks to the microbial population as a means to manipulate and/or control the retting process, with the intent to provide a cost effective means to increase consistency of the product. This experiment was conducted in order to learn how the microbial population responds to environmental conditions such as moisture variability and soil exposure, but controlled within a greenhouse rather than variable conditions in the field. Initially, we hypothesized that the bacterial population of the stalk at cutting would shift over time, correlating with under-retted and optimally-retted, as well as considerably over-retted material, and that treatments manipulating moisture and access to environmental microbiota would influence the population at these time points; however, the results indicate that changes in the retting population happen slowly, and significant treatment effects began to emerge only in the final time points.

The LM treatment, which received the least amount of water during retting, resulted in significant differences compared to HM and SHM, including lower richness and evenness at T6 and ANOSIM community similarity comparisons showing increasing R-values over time. While specific OTUs did not show strong associations in ISA or LeFse analysis by treatment, the HM and SHM samples showed a significant increase in the abundance of the phylum *Bacteroidetes* at T5 and T6, whereas the LM treatment did not show significant differences in abundance of phyla or genera over time. The addition of soil in the SHM treatment did not significantly impact the microbial community of the stalk compared to the other treatments, nor did it increase the speed of retting. Analysis of the soil used in the treatment

showed some overlap with respect to the most abundant bacteria found associated with the samples, but as there was little change from T1, in which no treatment had yet been applied to samples cut and removed from field, it seems likely that organisms associated with the hemp stalks were already present in/on the stalks at the time of cutting, and were not influenced greatly by exposure to soil throughout the retting process in this study. Varietal differences seen initially decreased over time—a possible explanation for the initial difference between SSa (lower alpha diversity) and the other two varieties is the stage of growth at harvest. While FE and FU varieties had flowered and were beginning senescence at harvest, the SSa had not flowered and was still in a stage of vegetative growth—a peculiarity of this experimental variety which was found not to flower at all under conditions from this field trial.

Our results indicate that a resilient microbial community associated with the plant stalks at harvest remained throughout the retting process and changed little according to the treatments applied and over the five weeks it took to ret the stalks completely. While the results do not support large shifts in the population according to time or treatment, a notable increase in the phylum *Bacteroidetes*, especially the genus *Chryseobacterium,* was observed in the final time points for HM and SHM treatments, but not LM, indicating moisture levels may influence this population over the course of retting. These results are in agreement with field studies of hemp and flax dew-retting, where similar microbial profiles were reported, and *Bacteroidetes* was found to increase toward the end of the retting process [15–17]. Previous analysis of the bacterial population changes that occur within retting hemp stalks showed an increase in the ratio of *Bacteroidetes* from mid-retting to full-retting conditions [18]. Flax, another natural fiber plant that relies on retting, also showed an increase in *Bacteroidetes* during the dew-retting process [15]. This phylum has previously been implicated in cellulolytic activity in both agricultural soils as well as gut microbiomes, and thus an increase in abundance of these organisms may signal a shift towards increased cellulose degradation and over-retting of fibers, following the depletion of pectin [18,19]. More research is needed to determine if higher abundance of *Bacteroidetes* correlates with fiber quality post-retting, nevertheless these results suggest a relationship between moisture levels during retting and abundance of this phylum at a crucial time in the retting process where the fiber is optimal, but before over-retting resulting in fiber degradation occurs. The relatively minor treatment effects on microbial diversity and community composition in this study did not appear to have affected retting time; all samples regardless of treatment were well-retted by T4/T5, and over-retted at T6.

Since the passing of the 2014 Farm Bill, opportunities for both growers and researchers interested in the potential of industrial hemp have increased in the US, although many obstacles remain. Specifically for fiber use, problems hinge around issues of scale and economy, in which the retting and decortication process has been described as a "bottleneck" for supplying consistent product to industry manufacturers [2,4,20,21]. Research addressing this problem has focused broadly on two areas: alternatives to field-retting such as solid-state fermentation with specific organisms and post-harvest treatments that focus on improving the end product without regard to the logistical and economic considerations, or adjusting agronomic practices such as timing of planting and harvest, turning in the field, and variety selection [6,15,17,22–24]. Fungal species are predicted to play a role in retting, by both their own degradation abilities and the process by which they facilitate entry of surface microorganisms past the cuticle, and have been a target for attempts to manipulate retting outcomes [17,25]. These efforts have all contributed useful information, but moving towards cost-effective retting practices that result in consistent fiber production with the specific qualities required by industry will require substantially more research from a variety of directions, such as traditional plant breeding for desirable field retting qualities (high cellulose, low lignin and pectin), molecular research into genes that affect fiber yield and quality, and microbiological investigations into specifically how the microbial profile associated with bast fiber crops is recruited and maintained throughout the plant's life as well as the retting process [20,26–29].

5. Conclusions

This work, together with past and recent studies of bast fiber retting, indicate that hemp (as well as other common bast fiber plants) harbor a resilient cohort of microorganisms that appear to be present at harvest and continue to persist throughout the retting process. To our knowledge, research into which microorganisms may be endophytes living within the stalks and which are surface dwelling has not been done. Endophytes are likely to be present in relatively low abundances at the time of harvest, and while significant patterns among the low abundance microorganisms were not detected, their contribution to the retting process cannot be discounted, as they are already "on-site" within the stalk when the plant is cut and the degradation begins. The resiliency of the bacterial community itself is ecologically interesting, and may provide a platform on which to study the concepts of resistance and resiliency in retting communities according to environmental disturbance and/or attempts to alter the community thorough applications of specific materials such as fungal/bacterial inoculum [30].

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Determination of tobacco alkaloid enantiomers using reversed phase UPLC/MS/MS

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ABSTRACT

N′-Nitrosonornicotine (NNN), a carcinogenic tobacco-specific N′-nitrosamine (TSNA), is on the FDA list of harmful and potentially harmful constituents (HPHCs). Nornicotine, a product of the demethylation of nicotine, is the immediate alkaloid precursor for NNN formation. Nicotine, nornicotine and NNN are optically active. The accumulation of the isomers of nicotine, nornicotine, and NNN impacts their biological activity. In this paper, we report the determination of tobacco alkaloid enantiomers (including nicotine, nornicotine, anabasine, and anatabine) in samples of different tobacco lines using a reversed phase ultra-performance liquid chromatography-tandem mass spectrometer (UPLC/MS/MS) method. Current method demonstates excellent detection capability for all alkaloid enantiomers, with correlation coefficients (r^2) > 0.996 within their linear dynamic ranges. The limit of detection (LOD) and limit of quantitation (LOQ) of all analytes are less than 10 ng/mL and 30 ng/mL, respectively. In addition, their recovery and coefficient of variation (CV%) are within 100–115% and 0.2–3.7%, respectively. The method validated in this paper is simple, fast, and sensitive for the quantification of alkaloid enantiomers in tobacco leaf and has been applied to investigations of tobacco alkaloid enantiomer ratios in different tobacco lines and tobacco products.

1. Introduction

Smoking damages many body organs and causes diseases to smokers. Cigarette smoking is the leading preventable cause of death in the United States. Worldwide, smoking causes approximately 7 million deaths each year [1]. In June 2009, the law authorized U.S. Food and Drug Administration (FDA) to regulate the manufacture, marketing, and distribution of tobacco and tobacco products. In March 2012, FDA established a list of harmful and potentially harmful constituents (HPHCs) in tobacco products and tobacco smoke [2]. N′-Nitrosonornicotine (NNN) is listed as a carcinogenic compound on the FDA HPHCs list. Nornicotine, the result of nicotine demethylation, is the immediate alkaloid precursor for NNN formation. Both nicotine and nornicotine exist as (R)- and (S)- enantiomeric isoforms that differ at the 2′-C position of the pyrrolidine ring (Fig. 1). The different alkaloid enantiomers have different pharmacological activities. It has been reported that (S)-nicotine is more pharmacologically potent than (R)-nicotine [3, 4] while (S)-NNN is more carcinogenic than the (R)-isoform [5]. Although (R)-nicotine shares many physicochemical properties with (S)-nicotine, it has been found that (S)-nicotine has a greater level of toxicity. LD_{50}s for intravenous administration of (R)-nicotine in several species of animals have been approximately 18 times higher than that of (S)-nicotine [4].

The carcinogenicity of (S)-NNN has been postulated to be greater than (R)-NNN in rat esophagus [6, 7], and this was confirmed by a recent rat feeding assay [5]. Since nornicotine is the major metabolite of nicotine and the precursor of NNN, and it has been verified that (S)-NNN is produced only from (S)-nornicotine [8], we may reduce the harmful effects of cigarettes by adjusting the enantiomeric ratio of nornicotine, and hence the enantiomeric ratio of NNN. More of the (R) form would be desirable. The enantiomeric composition of NNN is dependent on the enantiomeric composition of its alkaloid precursor, nornicotine [8]. Quantitative analysis of the enantiomers of nicotine, nornicotine, and NNN is crucial to understand the metabolomic mechanism and compositional changes in the enantiomers of nicotine and nornicotine, and thus the enantiomer composition changes of NNN and potentially its biological activity.

A few papers have reported the determination of alkaloid enantiomers in tobacco. Alkaloid enantiomer analyses have been performed using normal phase high-performance liquid chromatography (HPLC) coupled with a diode-array or UV detector, multi-dimensional gas

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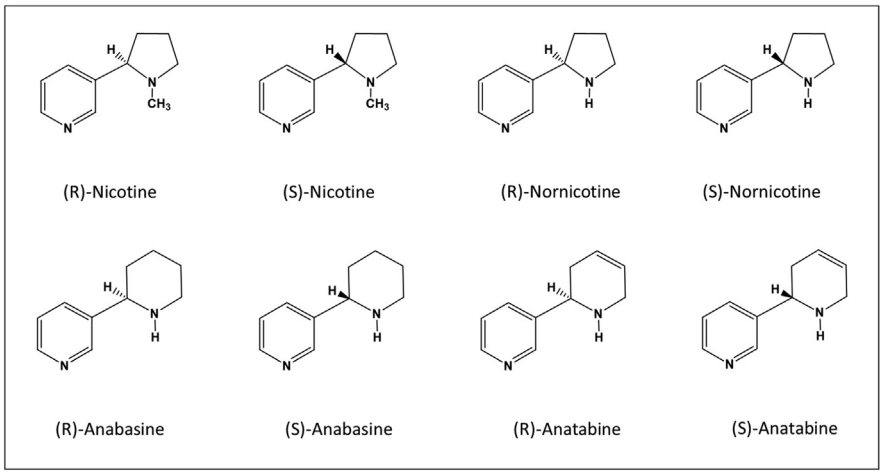

Fig. 1. Structures of tobacco alkaloid enantiomers.

chromatography with mass spectrometry, or combined HPLC-UV detector and GC/MS [9, 10, 11, 12, 13]. However, these methods have limitations for routine analysis. The GC method involves the derivatization of alkaloids and has a long analysis time. Normal phase HPLC uses toxic and strong organic solvents, thereby requiring instrumentation to have greater tolerance for organic solvents. In this paper, a novel reverse-phase UPLC/MS/MS method for determination of alkaloid enantiomers in tobacco is presented. The advantages of this method include simple sample preparation with the extraction of alkaloids using methanol and water, running HPLC analysis with regular mobile phases, as well as higher sensitivity comparing with existing methods. This method has been successfully applied to alkaloid enantiomers analysis in different tobacco lines and tobacco products.

2. Experimental

2.1. Samples

Reference ground tobacco products (burley, flue-cured, oriental, dark fire-cured, and dark air-cured tobacco leaf) were purchased from the Center for Tobacco Reference Products (University of Kentucky, KY). CORESTA smokeless tobacco reference products were obtained from North Carolina State University, Raleigh, NC. Plants of different tobacco lines were grown at the University of Kentucky Agricultural Spindletop Farm. Lines included (1) a high nicotine demethylation line of TN90, (2) low nicotine demethylation lines of TN86 and TN90, and (3) double and triple mutant lines of TN86 and TN90 for decreased nicotine demethylation. The triple mutant lines carry knockouts of genes CYP82E4, CYP82E5, and CYP82E10 for nicotine demethylation and the double mutant line carries knockouts of CYP82E4 and CYP82E5 [8]. The transgenic plants were RNAi knockouts of all nicotine dementylation genes in the TN90 line [14]. All tobacco plants were harvested and air-cured in a traditional air-curing barn. After curing, the fourth leaf from the top of each plant was removed, the lamina and midrib were separated, and then five leaves from different plants were combined, freeze-dried, and ground as one sample. There were four replicates for each sample.

2.2. Reagents and materials

The alkaloid analytical standards (racemic R, S-nicotine (purity ≥99%), racemic R, S-nornicotine (purity ≥98%), and racemic R, S-anabasine (purity ≥97%)), and ammonium formate (LC/MS grade, putrity ≥99.0%) were purchased from Sigma-Aldrich Corporation (St. Louis., MO). Racemic R, S-anatabine (purity ≥97%) was from Matrix Scientific (Columbia, SC). (R)-nicotine (purity ≥98%), (S)-nornicotine (purity ≥96%), and isotopically labeled racemic R, S-nicotine-d_4 (purity ≥98%) were obtained from Toronto Research Chemicals (Toronto, Canada). Labeled racemic R, S-nornicotine-d_4 (purity ≥98%) was from CDN Isotopes Inc. (Quebec, Canada). Nicotine-d_4 and nornicotine-d_4 isomers were used for internal standards. LC/MS grade acetonitrile and methanol were from Fisher Scientific (Hampton, NH). Tomato leaf (NIST 1573a) standard reference material was purchased from the National Institute for Standards and Technology (NIST) (Gaithersburg, MD). The tomato leaves were used as the matrix during the experiment because tomato and tobacco are members of the same plant family, *Solanaceae* [15], but tomato leaves do not contain detectable levels of tobacco alkaloids.

2.3. Instrumentation and apparatus

All analyses were done on a Waters ACQUITY UPLC H-Class System equipped with Xevo TQD Triple Quadrupole Mass Spectrometry (Waters Corporation; Milford, MA). The CHIRALPAK AGP column (150 × 4 mm column with 5 μm particle size) and LUX-Cellulose-2 column (150 × 2 mm column with 3 μm particle size) were used for nicotine and nornicotine enantiomer analysis, respectively. The enantiomers of anabasine and anatabine can be separated on both columns. Only the CHIRALPAK AGP column was used for analysis of anabasine and anatabine enantiomers in this paper. Separation of the enantiomers of nicotine, anabasine, and anatabine was achieved using an isocratic mobile phase program consisting of 90:10 (v/v) of 30 mM ammonium formate with 0.3% NH_4OH and methanol with a flow of 0.4 mL/min (Fig. 2). For nornicotine enantiomer separation, an isocratic program of 90:10 (v/v) of 20 mM ammonium formate with 0.2% NH_4OH:acetonitrile was used, and the flow rate was 0.2 mL/min (Fig. 2). The Waters Xevo TQD was operated in the electrospray ionization (ESI) in the positive mode with Multiple Reaction Monitoring (MRM) (Table 1). Source and desolvation temperatures were 150 and 500 °C, respectively. The desolvation gas flow was 800 L/hr. The capillary voltage was 0.38 kV.

2.4. Sample preparation and analysis procedure

Tobacco plant samples were freeze-dried and ground to pass through a 1 mm screen and mixed well to ensure homogeneity. 200 mg of ground tobacco samples were placed in 20 mL glass vials. Isotopes of racemic R, S nicotine-d_4 and racemic R, S-nornicotine-d_4 as internal standards were spiked into the same vial prior to the addition of 200 μL of 5N NaOH pre-treatment solution, and the samples were allowed to stand for 10 minutes. After that, 10 mL 70% methanol was added and the mixture was placed on a horizontal shaker to shake for

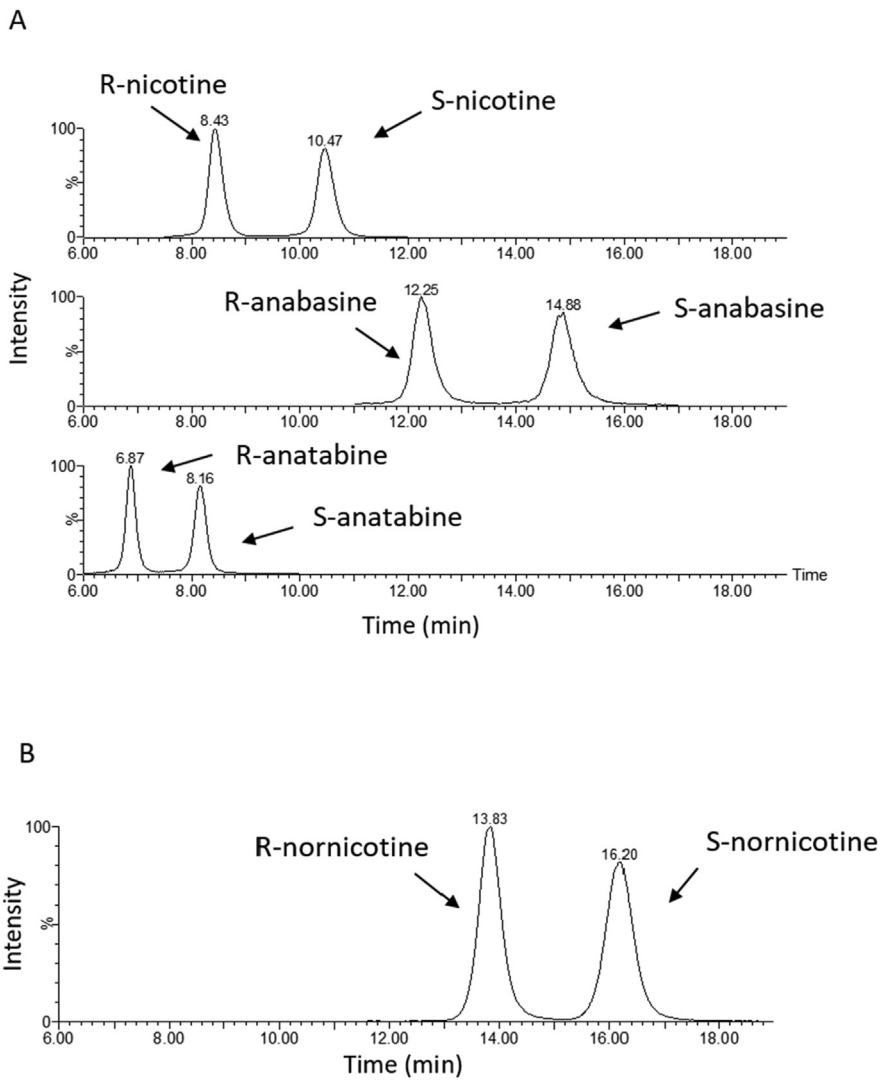

Fig. 2. Separation of nicotine, anatabine, and anabasine enantiomers from racemic alkaloid standards on the CHIRALPAK AGP column (A). R-nicotine and S-anatabine were distinguished and quantified by the differences in quantitation and confirmation transitions (m/z) for each (see Table 1). Separation of nornicotine enantiomers from the racemic nornicotine standard on the LUX Cellulose-2 column (B).

one hour. The extracts were filtered through a 0.22 μm PTFE filter to remove the tobacco powder and then diluted 20-fold with 70% methanol for alkaloid measurement. A 2 μL aliquot of the filtered extract solution was injected into the UPLC/MS/MS to determine the alkaloid enantiomers in each tobacco sample. For each tobacco sample, only one extraction needs to be done, but two injections onto the appropriate column were required to obtain the enantiomer results for all of the alkaloids.

3. Results and discussion

3.1. Column selection

There are many types of chiral columns on the market at present. The first step in method development was column screening. The CHIRALPAK AGP and CBH columns, LUX Cellulose-1, LUX Cellulose-2, LUX Cellulose-3, LUX Cellulose-4 and LUX Cellulose-5 columns were tested.

Table 1
Mass spectrometric parameters for the quantification and confirmation of alkaloids in the multiple reaction monitoring (MRM) mode.

Compound	Quantitation Transition (m/z)	Collision (V)	Confirmation Transition (m/z)	Collision (V)
(R)-Nicotine	163 > 132	16	163 > 117	26
(S)-Nicotine	163 > 80	14	163 > 117	26
(R,S)-Nicotine-d_4	167 > 136	14	167 > 121	24
(R,S)-Nornicotine	149 > 117	20	149 > 80	18
(R,S)-Nornicotine-d_4	153 > 121	22	153 > 96	24
(R,S)-Anabasine	163 > 92	20	163 > 80	18
(R,S)-Anatabine	161 > 107	12	161 > 80	28

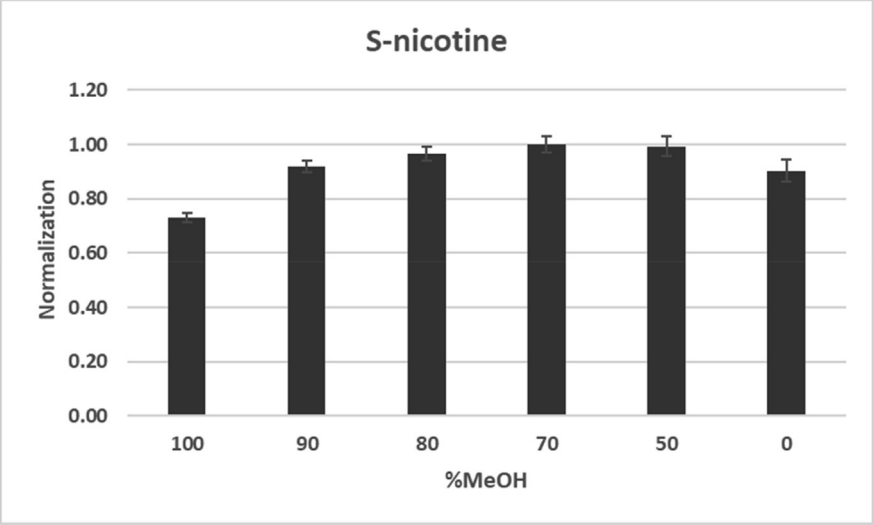

Fig. 3. Extraction efficiency of S-nicotine from ground tobacc in different concentrations of methanol (n = 5). Data were normalized to 70% MeOH extraction.

Table 2
Summary of the limits of detection (LODs), the limits of quantitation (LOQs), and calibration curve range/linearity (n = 5).

	LOD (ng/mL)	LOQ (ng/mL)	Calibration Range (ng/mL)	Slope (average)	Intercept (average)	Linearity, R^2 (average)
(R)-nicotine	8.4	28	100–4200	1.0651	-5.8554	0.9987
(S)-nicotine	5.4	18	350–14000	0.1437	-4.2977	0.9982
(R)-nornicotine	6.0	20	50–2100	1.1244	-5.3211	0.9988
(S)-nornicotine	6.0	20	50–2100	1.1177	-5.6773	0.9988
(R)-anatabine	4.8	16	50–2110	0.5545	-1.1916	0.9967
(S)-anatabine	5.7	19	50–2110	0.5630	-3.0739	0.9975
(R)-anabasine	1.2	4	15–530	0.8571	-0.8130	0.9984
(S)-anabasine	4.2	14	15–530	0.8223	0.3679	0.9991

Preliminary results indicated that the enantiomers of nicotine, anabasine, and anatabine had full baseline separation when the CHIRALPAK AGP column was used. However, the enantiomers of nornicotine only reached 85% baseline separation with this column. To obtain high resolution separation of nornicotine isomers, the LUX Cellulose-2 column was used (Fig. 2). Both columns separated anatabine and anabasine enantiomers very well, the resolution value is greater than 1.5.

3.2. Optimization of mobile phase

Different buffer solutions, including ammonium acetate, ammonium bicarbonate, and ammonium formate, were investigated for an appropriate mobile phase. The optimum result was achieved with ammonium formate. It was found that the pH value was critical for separation of the alkaloid enantiomers (data not shown). pH affects peak separation as well as the retention time. Ammonium hydroxide was used to adjust the pH to 9.5 for alkaloid enantiomer separation. Different organic solvents, such as acetonitrile, methanol, 2-propanal, and t-butanol were tested as mobile phase modifiers to improve the enantiomer separation. Our results indicate that methanol and acetonitrile are good mobile phase modifiers for the separation of nicotine and nornicotine enantiomers, respectively.

3.3. Optimization of extraction method

To optimize the extraction of alkaloids from ground tobacco leaf, the samples were extracted by shaking for one hour in different percentages of methanol and water. Mixtures consisting of 100%, 90%, 80%, 70%, 50%, and 0% methanol in water were evaluated. Five replicates were performed for each condition. The 70% and 50% methanol/water mixtures were found to have similar extraction efficiency for all target analytes. The S-nicotine extraction efficiency as an example was shown in Fig. 3. However, 50% methanol, which has greater water content, is capable of removing more salt from the tobacco samples and could potentially cause salt precipitation issues later. Therefore, 70% methanol was chosen as the extraction solution in these studies.

Table 3
Method accuracy with spiked alkaloids into the tomato leaf matrix (n = 3).

Analyte	Spiked Concentration (ng/mL)	Recovery %	CV %
(R)-nicotine	944.6	104.6	2.4
	1889.2	107.4	1.3
	3778.4	103.9	0.5
(S)-nicotine	944.6	108.8	3.3
	1889.2	106.2	0.7
	3778.4	104.9	1.9
(R)-nornicotine	428.8	114.5	3.7
	857.6	115.7	0.2
	1715.2	113.2	2.6
(S)-nornicotine	428.8	112.8	2.0
	857.6	113.4	1.1
	1715.2	109.0	1.1
(R)-anatabine	422.0	103.4	2.9
	844.0	104.8	1.4
	1688.0	102.7	0.4
(S)-anatabine	422.0	102.6	1.7
	844.0	103.4	1.3
	1688.0	101.9	0.7
(R)-anabasine	213.6	101.4	3.6
	427.2	104.0	1.0
	854.4	102.9	1.8
(S)-anabasine	213.6	107.2	3.5
	427.2	108.9	1.0
	854.4	107.3	1.8

Table 4
Percentage of R-alkaloids and demethylation of nicotine in the different tobacco lines (n = 4).

Sample	Type	Nicotine R/total %	Nornicotine R/total %	Anatabine R/total %	Anabasine R/total %	Nicotine Demethylation %
TN90	transgenic	4.11	16.93	9.13	33.95	0.58
	triple mutant	3.81	17.20	9.57	40.08	0.57
	double mutant	0.32	75.89	10.15	42.40	2.02
	Low Conversion	0.09	57.86	9.35	40.37	2.43
TN86	triple mutant	4.32	15.02	9.88	40.19	0.66
	Low Conversion	0.11	53.72	10.28	42.78	2.61
TN90	High Conversion	0.11	7.21	10.69	48.63	38.84

3.4. Method validation

The standard calibration curves of the alkaloid enantiomers were established by injecting a series of individual alkaloid enantiomer standard solutions of known concentrations into the UPLC/MS/MS. The standard calibration solutions of these alkaloid enantiomers were made by spiking a known amount of alkaloid enantiomers and internal standards (isotope racemic R, S nicotine-d_4 and racemic R, S nornicotine-d_4) in the tomato leaf matrix before following the sample extraction procedure mentioned above in the Experimental Section. A 2 μL aliquot of each filtered extract solution was injected into the UPLC/MS/MS. Masslynx software was used to collect and process the data. The ratios of the peak area of each enantiomer to their corresponding internal standard were calculated. The graphs of the peak area ratio versus the concentration ratio of each alkaloid to its appropriate internal standard were plotted. The calibration type is linear with 1/x weighting, and the regression lines are not forced through the origin. All alkaloid enantiomers showed an excellent linear response (>0.996) (Table 2).

The method was validated for precision and accuracy of each analyte at different concentrations. The low, medium, and high levels of the alkaloid isomers with internal standards were spiked onto the tomato leaf matrixes. Three replicates of each concentration level were tested. The blank tomato leaf with internal standards only was used as the control. The recovery and coefficient of variation (CV%) of each enantiomer in the extraction procedure was 100–115% and 0.2–3.7%, respectively (Table 3), which indicates that our method has good accuracy and precision. The limit of detection (LOD) and the limit of quantitation (LOQ) were estimated by injecting a series of known-concentration alkaloid enantiomer standards into the UPLC/MS/MS 5 times. The standard deviations of the concentration from the five injections versus the concentration of each alkaloid were plotted. The value of the y-intercept of a linear regression (s_0) is the estimation of standard deviation when the analyte is zero. The LOD and LOQ were estimated as $3s_0$ and $10s_0$, respectively [16]. The LOD and LOQ results are presented in Table 2.

3.5. Detection of enantiomers in tobacco leaf and tobacco products with current method

TSNAs are carcinogenic compounds present in tobacco leaf, and (S)-NNN has more toxicity than (R)-NNN [5]. In order to reduce the harmful effects of cigarette smoking, scientists have recently expended considerable effort to change the accumulation of nornicotine, the precursor of NNN, especially in burley tobacco. These efforts include the screening of parental seed source plants, the release of low nicotine demethylation (LC seed) varieties [17], and GMO tobacco lines [8]. Depending on the tobacco line, the percentage of R-nornicotine in total nornicotine varies from 4 to 75% [18]. Alkaloid enantiomer analysis can help elucidate the composition of these enantiomers in tobacco. Our reverse phase UPLC/MS/MS method has been used to identify and quantify the alkaloid enantiomers in different tobacco lines including low nicotine demethylation (low conversion, LC) and high nicotine demethylation tobacco samples, as well as double- and triple-gene mutants for lower nicotine demethylation, and RNAi transgenic plants. In these samples, the triple mutant and the RNAi transgenic tobacco samples have ultra-low levels of nicotine demethylation. Results from ultra-low nicotine demethylation tobaccos indicate that the ratio of nornicotine isomers is altered in the leaf. The ultra-low nicotine demethylation activity decreased the percentage of (R)-nornicotine in the leaf (Table 4). This is consistent with the previous results of Cai and Bush (2012) [18]. Our method was also used to measure the enantiomers of tobacco alkaloids in different kinds of tobacco samples, such as cigar filler, reference cigarette filler, CORESTA smokeless tobacco reference products, as well as different tobacco market types, including burley, oriental, dark air-cured, dark fire-cured, and flue-cured. Our results are consistent with other published data [10, 11, 12]. In these tobacco samples, the major enantiomer of nicotine is (S)-nicotine (>99%) and most of them have a higher percentage of (S)-nornicotine than (R)-nornicotine. The range of the percentage of (R)-nornicotine was 15–50%. The ratio of (R)- and (S)-anabasine was consistent in the different tobacco samples, and the levels of (S) anabasine were slightly higher than R-anabasine. The percentage of

Table 5
Percentage of R-alkaloids found in individual tobacco types, cigarette and cigar filler, and smokeless reference products (n = 3).

Sample	Type	Nicotine R/total %	Nornicotine R/total %	Anatabine R/total %	Anabasine R/total %
Tobacco leaf	RT2 (flue cured)	0.10	33.22	15.13	37.74
	RT3 (oriental)	0.38	14.94	15.72	41.97
	RT4 (Burley)	0.07	32.41	13.84	37.58
	RTDAC (dark air)	0.06	48.75	13.28	40.05
	RTDFC (dark fire)	0.40	40.14	13.42	40.76
Filler	RT6 (cigar filler)	0.27	16.24	13.43	38.67
	RT1 (1R6F filler)	0.13	30.32	14.65	38.60
Smokeless tobacco	CRP1.1 (Snus)	0.32	31.42	15.57	42.75
	CRP2.1 (Moist Snuff)	0.40	25.97	15.08	41.99
	CRP3.1 (Dry Snuff)	0.44	25.91	14.37	39.68
	CRP4.1 (Loose- leaf Chewing)	0.21	17.53	13.72	41.19

(R)-anatabine was also consistent in the different tobacoo samples, approximately 15% (Table 5).

4. Conclusions

We have developed and validated a simple, rapid, and sensitive UPLC/MS/MS method for the quantification of alkaloid enantiomers in tobacco samples. This reverse phase LC/MS/MS method has been successfully applied to the determination of tobacco alkaloid enantiomers in tobacco leaves as well as a wide range of tobacco products.

Declarations

Author contribution statement

Huihua Ji: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ying Wu: Performed the experiments; Analyzed and interpreted the data.

Franklin Fannin: Contributed reagents, materials, analysis tools or data.

Lowell Bush: Conceived and designed the experiments; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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REGULAR PAPER – PHYSIOLOGY/BIOCHEMISTRY/MOLECULAR AND CELLULAR BIOLOGY

Patterns of inheritance of acylsugar acyl groups in selected interspecific hybrids of genus *Nicotiana*

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Abstract
Glandular trichomes on the surface of Solanaceae species produce acyl sugars that are species-, and cultivar-specific. Acyl sugars are known to possess insecticidal, antibiotic, and hormone-like properties, and as such have great potential as a class of naturally occurring pesticides and antibiotics. The objective of this work was to analyze the acyl composition of acyl sugars in the leaf trichome exudate from selected *Nicotiana* species and to follow the inheritance of acyl content in their hybrids. Trichome exudates were collected, and the acyl profiles of acyl sugars were identified via GC–MS. The variations in acyl group inheritance in the hybrids (a single parent resemblance, missing, complementary, and novel groups) matched the patterns described in the literature for a variety of secondary metabolites. However, we did not find a complementation of major parental acyl groups. Instead, in some hybrids we observed a dynamic change in the proportions of acyl groups, distinguishing the acyl group profiles as novel. We observed paternal (i.e. *N. tabacum* cv. Turkish Samsun × *N. benthamiana* hybrids) and maternal (i.e. *N. tabacum* cv. Samsun-nn × *N. otophora*) inheritance patterns, novel acyl profiles (N. excelsior hybrids), and missing acyl groups (*N. excelsiana*). Selective inheritance of some acyl groups in the hybrids of *N. benthamiana* (4- and 5-methylheptanoic isomers) or *N. alata* (octanoate) was found. Suggestions are given to explain certain patterns of inheritance. The data presented here contribute to the body of knowledge about the effect of interspecific hybridization on the secondary metabolites by including acylsugar acyl groups that have not been studied previously.

Keywords Acylsugar acyl groups · Genus *Nicotiana* · Inheritance · *Nicotiana* hybrids · Trichomes

Introduction

Acyl sugars (ASs) are naturally-occurring secondary metabolites that are synthesized mainly by enzymes in the glandular trichomes on the surface of plants from the Solanaceae family. Much of the tobacco literature uses the term sugar esters (SEs) to describe acyl sugars. ASs are known to have important roles in plant defense against numerous pests and pathogens such as insects, aphids, whiteflies, budworms, and hornworms, bacteria and fungi (Cesio et al. 2006; Chortyk et al. 1993; Cutler et al. 1992; Leckie et al. 2012; Liu et al. 1996; Luu et al. 2017; Puterka et al. 2003; Severson et al. 1985a). ASs belong to the group of glycosides in which sucrose, glucose, or fructose moieties are esterified to fatty acids having short ($\leq C_6$) and/or medium carbon chain length (C_{7-12}), and either straight or branched chain. The structures of ASs and their acyl substitutions have been elucidated and presented in numerous publications (Arrendale et al. 1990; Kaiser et al. 2018; Luu et al. 2017; Severson et al. 1991). Species from the genus *Nicotiana* in the Solanaceae family include tobacco (*Nicotiana tabacum*) and wild tobacco relatives (Goodspeed 1954). The ASs of *N. tabacum* vary from those of the other *Nicotiana* species with respect to sugar moiety and acyl groups attached to them (Ashraf-Kohorassani et al. 2008; Jia et al. 2013; Leffingwell 1999; Severson et al. 1985a). Tobacco produces sucrose esters, where the esterified acyl groups are short-length ($\leq C_6$) branched-chain groups with a minor presence of C_7 groups.

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Three acyl groups are esterified to the glucose moiety, and an acetyl group may be attached to the glucose and/or fructose. The major acyl groups in burley, Virginia, and fluecured tobaccos are 2-methyl butyrate (2-MeBut), 3-methyl butyrate (3-MeBut), and acetate (Ac), and in oriental tobaccos the major groups are 3-methyl valerate (3-MeVal) and Ac (Ashraf-Kohorassani et al. 2008; Jia et al. 2013; Leffingwell 1999; Severson et al. 1985a). In some *Nicotiana* species such as *N. hesperis, N. occidentalis, N. alata, N. gosseii, and N. amplexicaulis*, glucose esters predominate (Jackson et al. 1998) or are present together with sucrose esters (Matsuzaki et al. 1989). Acyl groups in some wild *Nicotiana* species such as *N. glutinosa*, *N. occidentalis*, *N. obtusifolia*, and *N. alata* have up to 10 carbons, which form branched and/or straight chains (Chortyk et al. 1993; Cutler et al. 1992; Haliński and Stepnowski 2013; Kroumova et al. 2016; Nottingham et al. 1996).

ASs with medium-chain acyl groups ($C_{7–12}$) are more toxic than those with short-chain groups ($C_{2–6}$) to soft bodied arthropods such as budworms, hornworms, aphids, and whitefly (Chortyk et al. 1996; Jackson and Danehower 1996; Jackson et al. 1998; Nottingham et al 1996; Severson et al. 1991; Simonovska et al. 2006; Song et al. 2006) and bacteria (Chortyk et al. 1993; Cutler et al. 1992). Similarly, the medium-chain acyl groups 9-methyl nonanoate (9-MeNon) and decanoate (Dec) from wild tomatoes play an important role in insect resistance (Escobar-Bravo et al. 2016). Synthetic sucrose esters with octanoic acid (Oct) (C_8) have been successfully used against a range of arthropod species in tobacco (Song et al. 2006) and Florida citrus (Michaud and McKenzie 2004; Puterka et al. 2003).

Wild *Nicotiana* species are a great resource for genetic variability of the ASs. If the medium-chain acids can be introduced into the SEs of tobacco crops, they might enhance pest and insect resistance and consequently contribute to better quality and yield of tobacco leaves. One way of introducing new secondary metabolites is via hybridization. Plant hybridization is a known phenomenon. It is thought that around 70% of flowering plants originated via hybridization (Arnold 1994; Whitham et al. 1999). Hybridization can result in the introgression of traits, development of new species, and can influence the relationships between plants and their environment (Orians 2000). The effect of hybridization on the secondary product chemistry of hybrids and the impact on herbivore resistance has been the subject of several reviews (Cheng et al. 2011; Lopez-Caamal and Tovar-Sanchez 2014; Orians 2000; Rieseberg et al. 1993) summarizing 62 studies. The overall conclusion was that hybridization contributes to secondary metabolite diversity and affects herbivore resistance.

The genus *Nicotiana* comprises about 80 reported species, 57 of which have been shown to hybridize between themselves and with *N. tabacum* (Barbeć 2015; Lewis 2011). The interspecific crosses thus far have been estimated to number ~ 300 (Barbeć 2015). The abundance of successful interspecific crosses in this genus may be due to "slower evolution of genetic blocks to hybridization than the gene and chromosome alterations" (Goodspeed 1954). *Nicotiana* hybrids have been explored for a variety of reasons: (1) Initially they were used to study the phylogenetic relationships within *Nicotiana* (Barbeć 2015; Lewis 2011); (2) Hybridization is used to transfer useful genes from wild, undomesticated relatives into tobacco. These non-*tabacum* species have been explored for crop improvement due to their genetic variability and because some of them harbor novel disease-resistance genes (see Lewis 2011 and the references therein). (3) The cytoplasmic and organelle genomes of wild species have been successfully used as a source of male-sterile cytoplasm (CMS) to increase the efficiency of hybrid seed production of commercial cultivars (Lewis 2011 and the references therein). (4) One important application of *Nicotiana* hybrids is that they can be advantageous platforms for the production of animal vaccines (Ling et al. 2012; Rice et al. 2013). (5) Intensive hybridization is being conducted within *N. tabacum* cultivars and introductions to "develop new tobacco hybrid cultivars bearing high yield, good quality, multiple disease resistance, and broad industry acceptability" (Miller 2012 https://reeis.usda.gov/web/crisprojectpages/0227012-tobacco-breeding-and-genetics.html). (6) Tobacco hybrids have also been created as sources of genetic variations for trichome-produced secondary metabolites (Nielsen 1989).

Regardless of the broad-spectrum exploration of the *Nicotiana* hybrids, little attention had been paid to the inheritance of secondary metabolites produced by glandular trichomes, mainly diterpenes and ASs. As an exception, Nielsen (1989) quantitatively compared trichome-produced AS, together with terpenoids [*cis*-abienol, α- and β-cembratriene diols (α- and β-CBT-diols)] in tobacco hybrids and their parental species. In contrast, in the genus *Solanum* (former *Lycopersicon*) from the same family, intense research has been conducted on acylsugars in the hybrids of tomato. The role of ASs against mites and aphids was observed in tomato hybrids after introducing type IV trichomes, a major producer of ASs having 10 carbon acyl groups, from wild tomatoes (Escobar-Bravo et al. 2016; Goffreda et al. 1990; McDowell et al. 2011; Snyder and Carter 1984). Variation in type IV trichome density, and respectively the abundance of SE, among the individual hybrids correlated with the variation in mite response. Furthermore, hybrids between *L. esculentum* and *L. pennellii* were less infested with aphids (*Macrosiphum euphorbiae*) than the common tomato, due to the increased number of AS-producing trichomes and increased amount of ASs (Carter and Snyder 1985).

We were not able to find accessible literature wherein the acyl composition of acyl sugars in the interspecific

Nicotiana hybrids has been reported. Moreover, to our knowledge, introduction of trichome-based pest and insect resistance (due to synthesis of variable ASs) from non-*tabacum* species into tobacco has not yet been reported.

Majority of the studies related to ASs and AS acyl groups of *Nicotiana* (and other solanaceous) species were done on leaves (Ashraf-Khorasani et al. 2008; Chortyk et al. 1993; Leffingwell 1999; Matsuzaki et al. 1989, 1992; Severson et al. 1985b, 1985a; 1991; Simonovska et al. 2006). Historically, leaves of *Nicotiana* species have been explored because of their commercial use and high-level accumulation of trichome exudate (5–30% of dry weight of leaves) playing important role in plant–insect, –microbe interaction (Wagner 1991). Here we analyzed trichome exudates from leaves of selected artificial *Nicotiana* hybrids and their parents for the presence of novel and variable ASs having medium chain-length, branched and straight-chain acyl groups along with short-chain groups to allow further tests for insecticidal potential. The ASs showing insecticidal properties can be used as templates for biotechnological design of acyl sugars that will improve pest resistance of *Nicotiana* crops. The variation of AS acyl group inheritance in the hybrids, such as complementary group inheritance, unidirectional inheritance from one of the parents, novel AS acyl group profile, and missing groups, were similar to the patterns described in the literature for a variety of secondary metabolites, including essential oils and phenolic glucosides (Cheng et al. 2011). An interesting phenomenon observed was the selective inheritance of certain acyl groups in the hybrids of *N. benthamiana* or *N. alata*. The data accumulated here contributes to the body of knowledge of the effect of hybridization on the secondary metabolites by including AS acyl groups that have not been previously studied.

Materials and methods

Plant materials

In 2000–2003, numerous interspecific crosses between commercial tobacco (maternal/paternal) and non-*tabacum* species (maternal/paternal) were performed at Kentucky Tobacco Research and Development Center (KTRDC) (Lexington, KY, USA) to identify interspecific hybrids that could be appropriate for the large-scale production of economically important proteins and that had resistance to common tobacco diseases. Hybrid seeds were created via cross pollination, as described in detail in Zaitlin and Mundell (2006). Hybrid #s H110, H114, 37, 87, and H75, were selected due to their potential to have diverse set of acyl groups coming from both parents (Table 1). The horticultural *Nicotiana* hybrids 'Baby Bella', 'Tinkerbell' and 'Whisper' were purchased from Swallowtail Garden Seeds, CA. Seeds from *N. excelsior* (KTRDC accession numbers 11-S-19-1 and 90-S-19-2), *N. benthamiana* (accession number S-6–5), and dark-type tobacco KY 171 were obtained from the KTRDC germplasm collection. *N. excelsiana* hybrid seeds were provided by Kentucky BioProcessing, Owensboro, KY, USA. *N. tabacum* cv. Samsun-nn (PI 552748), and *N. tomentosiformis* (PI 555572) seeds were obtained from the NPGS/GRIN collection. Flue cured Virginia K326 tobacco seeds were kindly provided by F.W. Rickard Seeds, Inc. (Winchester, KY, USA). Seeds

Table 1 Selected interspecific hybrids and possible major AS acyl group inheritance from both parents

Hybrid number	Maternal species	Major acyl groups	Pollen from:	Major acyl groups	Hypothesized major acyl groups in the hybrids' ASs
H110/H114	*N.t.* cv. Samsun-nn	3-MeVal[1,2]	×*N. benthamiana*	MeHept[a,3,4,5]	3-MeVal + MeHept
H75	*N. t.* SN 2108 (dark)	MeBut[b,1]	×*N. otophora*	MeHex[c], 3-MeVal[6]	MeBut, MeHex, 3-MeVal
H87	*N.t.* NC 297 (flue-cured)	MeBut[1]	×*N. otophora*	MeHex, 3-MeVal[6]	MeBut, MeHex, 3-MeVal
H37	*N. excelsior*	Oct, 5-MeHept, Hept[7]	×*N. bigelovii*	3-MeVal[3,6]	Oct, 5-MeHept, 3-MeVal

[1]Severson et al. (1985a)

[2]Leffingwell 1999

[3]Severson et al. 1991b

[4]Matsuzaki et al. 1992

[5]Chortyk et al. 1993

[6]Matsuzaki et al. 1989

[7]This work

[a]MeHept includes both—5- and 6-MeHept isomers

[b]MeBut includes both 2- and 3-MetBut

[c]MeHex includes both 4-and 5-MeHex

of *Alatae* hybrids were a gift from Dr. Catherine Poisson (Bergerac Seed and Breeding, France).

Hybrid seeds' germination

Hybrid seeds from the KTRDC hybrid seed collection were sterilized by incubation in 70% ethanol for one minute, followed by a 10-min incubation in 20% household bleach, (0.3% sodium hypochlorite aqueous solution) followed by rinsing five times in sterile water to remove all traces of bleach and ethanol. The seeds were then germinated in 95 × 15 mm Petri dishes on agar medium containing Murashige and Skoog (1962) (MS) salts and micro-elements (PlantMedia), Gamborg vitamins (1 ml) (Sigma-Aldrich Co. Ltd.) and high-strength agar (0.56%) (RPI Corp). Approximately one month later, rooted plantlets were transplanted to Styrofoam float trays containing a medium specially formulated for tobacco (Carolina Choice Peat-Lite Mix). Ten days later fertilizer (Peters 20-10-20, 1.5 g l^{-1}) was added, and the solution was replaced weekly thereafter. Plants were grown there for 3–4 weeks, whereupon those with well-established roots were transplanted individually into 4-inch pots. Seeds of the horticultural hybrids were sown on Pro-Mix medium (Premier Horticulture Inc., Canada) in 4-inch pots that were then covered with plastic until germination occurred in a growth chamber. All plants were grown at 22.8 °C with a 16 h/8 h light/dark regime and 23% humidity. After germination, the plastic covers were removed and plants were allowed to grow under the same conditions.

Collection of trichome exudate, butyl ester preparation, and GC–MS analysis

Trichome exudate was collected from the mid-stem leaves. Collection was done when plants were at a vegetative stage, usually three months post-sowing. The maturation time (the time of flower production) of hybrid plants of different crosses varied, but that of the F1 hybrid plants from the same cross were similar. We have continuously observed that around three months post-sowing the AS acyl composition is stable. Extract collection and AS acyl derivatization with butanol were conducted as described in Severson et al. (1985b) with some modifications. Abundance of SEs varied among species. Four or 8 discs (1 cm radius) were washed for 1 min with CH_3CN, with these washes vacuum-evaporated to dryness, dissolved in $CHCl_3$ (1 ml), transferred to 1.5 ml GC vials and dried. Where SEs were in low abundance (e.g. H37 and *N. excelsiana*), whole leaves were washed with CH_3CN for 1 min, the washes were vacuum-evaporated to dryness and dissolved in $CHCl_3$ (10 ml). Polar impurities were removed by twice partitioning with H_2O. Chloroform fractions were evaporated to dryness in GC vials (1.5 ml) (Kroumova et al. 2016). Further saponification, butyl ester derivatization of acyl groups, GC–MS analysis and acyl identification were completed as described previously (Kroumova et al. 2016). AS acyl groups were analyzed as butyl esters. The linear structure of acyl groups detected from all species and hybrids, their full and abbreviated names are given in Fig. S1. For the comparison of major acyl groups between hybrids and their parental species, peak areas were converted to moles using the linear regression equations derived from the calibration curves of standards (Kroumova et al. 2016). In the tables and in the graph the relative abundance of AS acyl groups of the individual plant types was calculated as mol% of total acyl groups.

Results

Selection of artificial hybrids

Hybrids from the *Nicotiana* collection KTRDC were selected on the basis of parental origin. *N. tabacum* was a preferred maternal line, in order to have hybrids with good biomass (Ling et al. 2012; Zaitlin and Mundell 2006). The other parent was selected because of its biosynthesis of medium length, branched, and straight-chain acyl groups (Table 1). We hypothesized that ASs produced by these hybrids may incorporate acyl groups from both parents. The major acyl groups in hybrids were postulated on the basis of the reviews showing that typically the F_1 hybrids express parental secondary metabolites (Cheng et al. 2011; Orians 2000). The non-*tabacum* interspecific hybrids *N. excelsior* × *N. bigelovii* were selected for the potential to combine 3-MeVal with straight-chain Oct and heptanoic acid (Hept), and medium-length branched-chain methyl heptanoic isomers (Table 1). Hybrid seeds H110 and H114 were produced by two independent hybridization events using the same parents. The same applies to the hybrids H75 and H87.

AS acyl group profiles

N. tabacum as an example of natural hybrid between *N. sylvestris* and *N. tomentosiformis*

An example of natural hybridization, the species *N. tabacum* originated from an interspecific hybrid between an ancestor of *N. sylvestris* and a particular ancestral lineage of *N. tomentosiformis* (and possibly *N. otophora*), that evolved hundreds of thousands of years after polyploidization (Gray et al. 1974; Lewis 2011). Table 2 compares the acyl composition of the modern parental species and two modern varieties of tobacco—oriental tobacco cv. Samsun-nn and the flue-cured Virginia tobacco cultivar K326.

Table 2 AS acyl group compositions of two commercial tobacco cultivars and their progenitor species

Acyl group	*N. sylvestris*[1]♀	*N. tomentosiformis*♂	*N. t.* Samsun nn (oriental type)	*N. t.* K326 (flue-cured)
	mol% of total			
Ac	64.3	25.0	30.9	36.5
MePro	–	1.0	9.0	6.6
But	6.6	1.9	4.5	2.0
2-MeBut	4.8	5.9	11.2	24.6
3-MeBut	1.0	3.4	10.5	19.2
3-MeVal	1.9	61.3	33.9	2.6
4-MeVal	1.0	1.3	tr	1.2
4-MeHex	15.6	–	–	2.8
5-MeHex	4.9	–	–	1.3
5-MeHept	–	–	–	1.5
6-MeHept	–	–	–	1.6

The data in each column represent average of three individually analyzed plants. The underlined acyl groups are the most abundant. The numbers represent mol% of total

– not observed, *tr* trace amount

[1]The data is from Kroumova et al. (2016)

In *N. sylvestris* the major acyl group is acetate, followed by 4-methyl hexanoate (4-MeHex); 3-MeVal is a minor acyl group. *N. tomentosiformis* SEs contain high amounts of 3-MeVal, followed by acetate and 2-MeBut. The acyl composition data for *N. tomentosiformis* is very similar to that already published by Severson et al. (1985b). Samsun-nn has the typical oriental-type profile, where 3-MeVal is the major group, followed by Ac and 2- and 3-MeBut, while Virginia K326 (air-cured type) tobacco has mostly Ac and 2-and 3-MeBut. AS acyl composition data for Samsun-nn and Virginia K326 presented here are in agreement with the literature (Kallianos 1976; Leffingwell 1999; Severson et al. 1985b). With respect to the presence of 3-MeVal as a major acyl group and absence of MeHex isomers, Samsun-nn resembles *N. tomentosiformis*. In Virginia K326, 3-MeVal is substantially reduced, but 2-and 3-MeBut that are present at lower abundances in the parental SEs became second and third major groups, after the Ac (Table 2). We suggest that a mutation in the isopropylmalate synthase gene (IPMS) (reaction 1, Fig. S3) in Virginia causes accumulation of precursor molecules and increased synthesis of 2-MeBut. Other changes are involved as well, leading to the final acyl profile. The presence of 3-MeVal as a major group in both *N. tomentosiformis* and *N. tabacum* Samsun-nn, and the lack of MeHex isomers favors the paternity of *N. tomentosiformis* vs. *N. otophora*, where MeHex isomers are the major groups (see *N. otophora* data in Table 4).

Artificial *N. tabacum* hybrids

N. tabacum cv. Samsun-nn × *N. benthamiana* (H110 and H114)

These hybrids were generated from two independent hybridization events using the same parents. The phenotypes of the hybrids are shown in Fig. 1a, b. Three plants from each hybrid were analyzed, and the data are presented in Table 3. The acyl compositions among individual plants of each cross were consistent, with some variation detected in the relative amounts. The acyl profiles consisted mainly of 6- and 5-MeHept, with small amounts of 5- and 4-MeHex. The other groups were minor. The similarity among AS acyl profiles of individual plants from either hybrid is not surprising because the F_1 plants were derived from the same hybridization event. What is more interesting is that the acyl composition from the second, independent cross of the same species gave a very similar qualitative acyl profile. We would expect some variations, due to the stochastic separation of chromosomes during the meiosis in both parental species, and respectively different combination of allelic chromosomes. The data from the three individual hybrid plants from each cross are compared with the parental compositions data (Table 3). Parental AS acyl group profiles reported here are in agreement with the published literature (Chortyk et al. 1993; Matsuzaki et al. 1992; Severson et al. 1991). The maternal donor—*N. tabacum* cv. Samsun-nn synthesizes predominantly 3-MeVal and 3-MeBut, while *N. benthamiana* (paternal donor) makes predominantly 5-and 6-MeHept and the minor acyl groups 4-MeHex and 5-methyl hexanoate (5-MeHex) (Table 3). The hybrids' AS acyl group profiles matched the paternal *N. benthamiana* profile—with a prevalence of 5- and 6-MeHept acids (90%) and small amounts of 4- and 5-MeHex. All parental groups were present in the hybrids, but no combination of the major acyl groups 3-MeVal and MeHept isomers occurred. 3-MeVal was present as a minor group.

N. tabacum × *N. otophora* hybrids (H75 and H87)

The maternal parent of hybrid H75 is a 'dark type' tobacco cv. SN2108, and the maternal parent of hybrid H87 is the flue-cured tobacco cv. NC 297 (Table 1). The phenotype of the hybrids is shown in Fig. 1c, d. The AS acyl composition of the hybrids and the parental species is presented in Table 4. *N. tabacum* KY171 (dark type) and *N. tabacum* K326 (flue cured) shown in Table 4 are not the exact maternal species, but they are representatives of the dark and flue-cured class of tobaccos. For each hybrid, three individual plants were analyzed. Compositionally, the acyl profile was very similar among the F_1 plants of the same hybrid and between the individuals of the two hybrids.

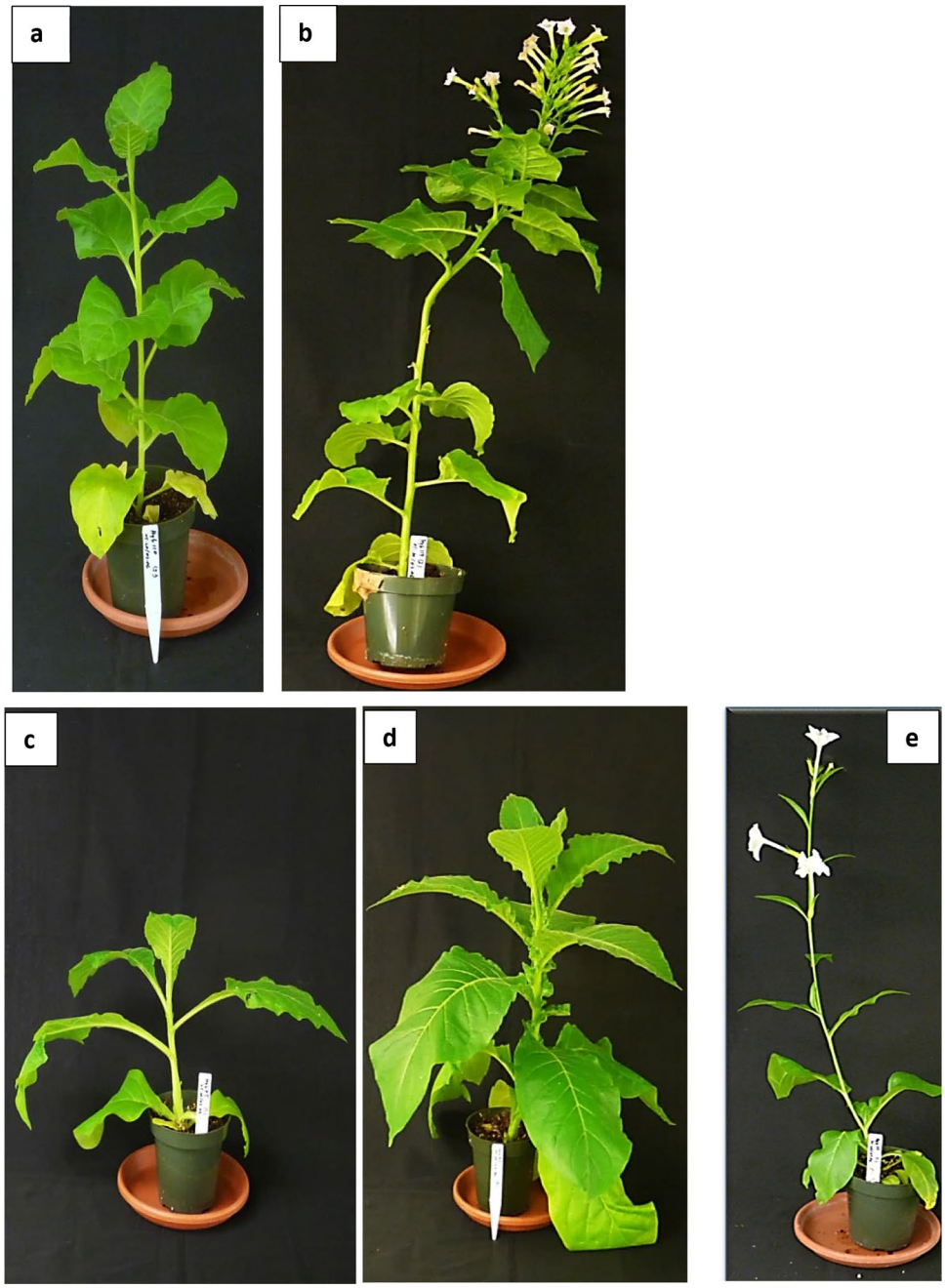

Fig. 1 Phenotypes of two *N. tabacum* cv. Samsun-nn × *N. benthamiana* F1 hybrids, of two *N. tabacum* × *N. otophora* F1 hybrids, and of *N. excelsior* × *N. bigelovii* F1 hybrid

3-MeVal was the predominant group, followed by 3- and 2-MeBut. There was some variability in the relative abundance among the individual plants of each hybrid. Still, when comparing the average values of individual acyl groups and their total distribution it is clear that there is a great similarity in ASs acyl group composition between the two hybrids (Table 4). The acyl profiles of the maternal species—dark tobacco (for H75) and flue-cured (for H87) tobaccos are also similar. Dark and flue-cured tobaccos were previously shown to produce ASs containing mostly 2-and 3-MeBut and Ac (Leffingwell 1999; Severson et al. 1985a). The major AS acyl groups of *N. otophora* are 4- and 5-MeHex and 3-MeVal (in declining order) (Severson et al. 1991). All parental acyl groups were inherited in the hybrids. 3-MeVal became a major group followed by small amounts of 2- and 3-MeBut. Some F_1 plants showed elevated abundances of MeHept isomers that otherwise were minor groups in N.t. K326 or were not detected in N.t. KY171. Thus, by crossing dark or flue-cured tobacco with *N. otophora*, the hybrids acquired novel AS acyl chemistry resembling oriental-type tobacco profile, but with additional eight-carbon branched-chain groups and missing Ac groups (Table 4).

Table 3 Comparison of the major AS acyl groups in two interspecific hybrids and the parental species

Acyl group	*N. t.* Samsun-nn	*N. benth.*	H110			H114		
	♀	♂	1	2	3	1	2	3
		mol% of total						
Ac	32.4	16.2	0.4	–	1.3	–	–	–
MePro	9.4	7.7	0.8	–	1.6	0.9	–	–
2-MeBut	11.7	0.6	0.3	–	0.5	0.5	tr	tr
3-MeBut	11.0	0.2	0.6	–	1.2	1.2	0.6	tr
3-MeVal	35.5	1.1	2.2	–	2.4	4.0	1.5	1.3
4-MeHex	–	1.8	2.8	1.9	2.7	2.5	2.2	1.8
5-MeHex	–	4.5	6.5	5.6	6.3	8.1	8.7	6.8
5-MeHept	–	31.4	30.6	27.7	29.2	25.1	23.8	22.7
6-MeHept	–	36.6	55.6	64.7	54.9	57.6	63.2	67.4

The data of the parental species represent average from three plants. Three individual F1 plants are shown for each hybrid. Underlined acyl groups are the most abundant. The numbers represent mol% of total

– not observed, *tr* races

Table 4 AS acyl composition of Hybrids 75 and 87 and their parental species

Acyl group	*N. t.* KY171[c] ♀	*N. otophora*[1] ♂	H75			H87			*N.t.* K326[d] ♀
	(Dark type)		1	2	3	1	2	3	(Flue cured)
			mol% of total						
Ac	47.5		–	–	6.7	–	–	–	36.5
MePro	3.8		1.1	–	0.7	3.3	–	2.2	6.6
But	0.9		0.5	–	0.3	0.5	–	–	2.0
2-MeBut	24.3		9.5	4.3	5.3	14.4	4.8	9.0	24.6
3-MeBut	15.0		11.8	6.4	7.4	12.5	4.9	8.0	19.2
3-MeVal	5.8	+[b]	70.9	67.2	67.5	58.9	82.1	44.6	2.6
4-MeVal	–		2.8	2.6	3.4	2.0	2.5	1.6	1.2[a]
4-MeHex	1.4[a]	+++[b]	0.8	1.0	0.9	0.9	1.0	1.7	1.3[a]
5-MeHex	0.5[a]	++[b]	–	1.4	1.1	0.8	–	2.5	2.8[a]
5-MeHept	–		0.9	4.7	4.0	2.4	1.8	7.8	1.6[a]
6-MeHept	–		1.6	12.4	2.7	4.4	3.0	22.6	1.5[a]

The data for the dark tobacco are averaged from two plants. The data for the flue-cured tobacco are averaged from three plants. Three individual F1 plants are shown for each hybrid. Underlined acyl groups are the most abundant. The numbers represent mol% of total

– not detected

[a]Peak abundance is low andmay be missed if small samples are taken for GC analysis

[1,b]The data is from Severson et al.(1991) where acyl groups are not quantified but the order of abundance is given

[c]*N. t.* KY171 is not the exact maternal parent for H75, but it is representative of dark type tobaccos

[d]*N. t.* K326 isnot the exact maternal parent for H87, but it is representative of flue-cured tobaccos

Artificial hybrids of *N. excelsior*

Hybrid H37

This hybrid is a cross between *N. excelsior* and *N. bigelovii*. The maternal parent, *N. excelsior,* is a leafy herb with mostly glabrous leaves and stems (https://www.flora.sa.gov.au/efsa/lucid/Solanaceae/Nicotiana%20species/key/Australian%20Nicotiana%20species/Media/Html/Nicotiana_excelsior.htm). The paternal species, *N. bigelovii* (or *N. quadrivalvis*) (Knapp et al. 2004) is a bushy herb, with small-lamina leaves and numerous glandular trichomes (https://www.nathistoc.bio.uci.edu/plants/Solanaceae/Nicotiana%20quadrivalvis.htm). The hybrid plants were small in size with mostly glabrous leaves, resembling the maternal parent (Fig. 1e). The hybrids produced very little exudate as judged by the slight leaf stickiness and by the low relative abundance as revealed by GC–MS. The acyl composition was similar among the

individual F_1 plants, comprising mostly 5- and 6-MeHept, followed by MeHex isomers and 3-MeVal. Comparison of the AS acyl groups between the parents and hybrids is shown in Table 5. We possessed two accession of *N. excelsior*. The precise maternal accession of Hybrid 37 was not given. That's why we analyzed both accession. High similarity of the acyl profile was observed between them with some differences in the proportion of groups (Table 5). *N. excelsior* SEs contained mostly Oct, 5-MeHept, Hept, and 2-MeBut while *N. bigelovii* synthesized mostly 3-MeVal, 2-MeBut, and Ac (Matsuzaki et al. 1989). Synthesis of the straight-chain groups Oct and Hept was not transferred maternally to the hybrids. Qualitatively, the acyl composition of F1 plants was closer to the paternal species *N. bigelovii*. However, the relative abundance of individual acyl groups varied substantially from *N. bigelovii*, which resulted in a novel acyl profile (Table 5). This profile showed substantial presence of medium length, branched-chain groups (~ 75% of all acyl groups). Variability was observed among the individual hybrid plants. More study is needed to address this variability. Regardless of the extract's insecticidal potential due to the predominance of medium-length, branched-chain (MeHept and MeHex) isomers, the scarce yield makes the extract unsuitable for further examination. However, the exudate of the maternal species, *N. excelsior*, can be explored further as the acyl profile consists mostly of MeHex isomers and medium-length straight-chain groups (> 72% of total acyl groups).

N. excelsiana

N. excelsiana is a fertile tetraploid hybrid derived from crossing *N. excelsior* and *N. benthamiana* that was created for the purpose of vaccine production (i.e. Ling et al. 2012). Like the maternal species *N. excelsior*, the hybrid is leafy with mostly glabrous leaves (Smith et al. 2009). The only AS acyl groups observed were 5- and 6-MeHept (Table 5). The comparison between parental species and *N. excelsiana* showed that the 6-MeHept group was inherited paternally from *N. benthamiana*, but 5-MeHept may have come from either parent. The straight-chain groups Hept and Oct from *N. excelsior* were not inherited in the hybrid. The acyl profile of ASs from *N. excelsiana* can be considered novel, consisting only of two acyl groups. The *N. excelsiana* AS acyl profile shows an example of missing secondary metabolites (Table 5). The trichome extract would be a good source for testing the insecticidal potential of MeHept isomers provided that a sufficient amount of SEs could be collected.

Artificial interspecific hybrids from section *Alatae*

N. × *sanderae*

N. × *sanderae* originated from a cross between *N. alata* and *N. forgetiana* (Chase et al. 2003). Images of this hybrid can be found on the web (https://www.alamy.com/flowering-tobacco-nicotiana-x-sanderae-nicotiana-sanderae-potte

Table 5 AS acyl compositions of *N. excelsior* interspecific hybrids and their parental species

Acyl group	*N. excelsior*♀		*N. bigelovii*[1]	Hybrid 37			*N. benthamiana*	*N. excelsiana*[a]
	11-S-19-1	90-S-19-2	♂	1	2	3	♂	
				mol% of total				
Ac	1.3	8.6	17.0	9.6	6.5	1.5	16.0	–
MePro	1.6	2.2	9.6	3.8	4.6	3.4	7.6	–
But	1.0	1.3	0.1	1.1	3.1	2.2	0.1	–
2-MeBut	7.3	13.6	19.5	1.6	7.6	4.3	0.6	–
3-MeBut	0.4	1.7	5.4	2.1	6.0	+	0.2	–
3-MeVal	0.7	–	45.9	5.1	20.5	5.2	1.1	–
4-MeVal	–	–	–	1.4	1.6	2.1	0.9	–
4-MeHex	tr	tr	0.1	4.0	7.7	10.0	1.8	–
5-MeHex	tr	tr	0.3	7.1	8.0	11.8	4.4	–
5-MeHept	31.3	15.9	0.6	21.8	19.8	30.8	31.1	36.9
6-MeHept	3.7	1.2	1.6	42.5	14.5	28.6	36.2	63.1
Hept	14.5	6.8	–	–	–	–	–	–
Oct	38.2	48.7	–	–	–	–	–	–

The data for the parental species are averaged from three plants. Three individual H37 F1 plants are shown. The underlined acyl groups are the most abundant. The numbers represent mol% of total

– not observed, *tr* traces

[a]The data was generated from pooled leaf extract from several plants

[1]The data is from Matsuzaki et al. (1989)

d-plants-image9257554.html). *N. alata* is the species from which most of the modern horticulture hybrids are derived (https://portlandnursery.com/plants/annuals/Nicotiana.shtml). *N. alata* synthesizes straight-chain, short and medium length acyl groups with up to eight carbons, and also short length, branched-chain, acyl groups with up to six-carbons (Table 6). In contrast, *N. forgetiana* has predominantly branched-chain groups with up to seven carbons (Severson et al. 1991). The comparison of *N.*×*sanderae* and its parents is shown in Table 6. The hybrid's major groups are Oct, 2-MeBut, and Ac, followed by Hex. The maternal species *N. alata* produces the same major groups. Straight-chain groups Hex and Oct are inherited from *N. alata,* but Ac and 2-MeBut may have been inherited from either parent. MeHex isomers that are the second and third major acyl groups of *N. forgetiana* were not detected in the hybrid. The *N.*×*sanderae* AS acyl group composition was confirmed, and AS structures were elucidated by British American Tobacco, Inc., Souza Cruz, Brazil (Guilherme Sabin, personal communication).

Alatae hybrids

The *Alatae* hybrids were created by Catherine Poisson (Bergerac Seed & Breeding SC, France) by crossing two inbred lines. These lines originated from a breeding program that started by crossing wild species from section *Alatae* (*N. langsdorffii, N. alata, N. forgetiana, N.* × *sanderae*), but for some of them commercial varieties were additionally involved. However, the hybrids are not direct progenies of the original wild species and commercial varieties, as many crosses and backcrosses have been done through the years (Poisson Catherine, personal communication). We analyzed 15 hybrid lines (Fig. S2) and found their AS acyl group compositions to be similar. In hybrids PO# 67, 15, 69, 70, 81, 101, and 120, Oct was the predominant group (> 50% of total acyl groups) (Fig. S2, Table 6) and the other groups were ≤ 10% each. In hybrids PO# 5, 33, 71, 72, 94, and 96, Oct was the major group followed by 2-MeBut. In all hybrids there was a presence of other then C_8 straight-chain groups (C_4, C_6, C_7, and C_9), and the total relative abundance of straight-chain groups in all hybrids was 60–90%. 2- and 3-Mebut were absent in POs 70, 111 and 112. There was variation in the relative acyl abundance between individual plants of each PO hybrid, but nevertheless the acyl profiles of the lines were well defined. The composition of PO5, 33, 71, 72, 94, and 96 hybrids was closer to that of *N. sanderae* and *N. langsdorffii* (Table 6). In section *Alatae* only *N. alata* and *N. langsdorffii* synthesize Oct, and therefore this acyl group could have been inherited from either of the species.

Table 6 AS acyl composition of *Alatae* hybrids and their parental species

Acyl group	*N. alata*[1]	*N. forgetiana*[2]	*N.*×*sanderae*[1]	PO67[a]			PO72[b]			*N.langsdorfii*[3]
	♀	♂		1	2	3	1	2	3	
			mol% of total							
Ac	23.9		21.5	–	–	–	–	–	-	
MePro	14.3		3.8	4.2	3.4	5.0	6.6	7.1	5.7	+
But	7.4		3.6	–	–	–	4.0	4.8	4.2	
2-MeBut	20.4	+++	30.2	4.3	5.5	4.9	23.2	35.9	23.1	+
3-MeBut	6.9		0.9	3.7	3.4	–	4.2	2.4	-	
3-MeVal	3.9		1.9	–	–	–	–	–	-	
Hex	6.2		3.5	–	5.0	–	6.8	5.4	5.7	
4-MeHex	–	+	–	–	–	–	–	–	-	
5-MeHex	–	+	–	–	–	–	–	–	-	
Hept	–		–	–	4.7	6.7	5.8	2.9	3.6	
Oct	16.8		34.7	87.8	73.0	83.4	44.0	38.9	54.4	+++
Non	–		–	4.2	5.0	–	5.2	2.6	3.3	

The data for *N. alata* and *N.*×*sanderae* are averaged from three plants. Three individual plants from two representative hybrids are shown. All species in the table were involved in the creation of the PO hybrids. The underlined acyl groups are the most abundant. The numbers represent mol% of total

– not observed

+ not quantified, but 2-MeBut in *N. forgetiana* and Oct in *N. langsdorfii* are designated as major groups

[1]Kroumova et al. (2016)

[2]Severson et al. (1991)

[3]Cutler et al. (1992)

[a]SE acyl composition of PO67 is similar to that of POs 15, 69, 70, 81, 101, and 120

[b]SE acyl composition of PO72 is similar to that of POs 5, 33, 71, 80, 94, and 96

MeHex isomers from *N. forgetiana* were not inherited during the breeding process. Acetate was not inherited from *N. alata* or *N.* × *sanderae* as well. Hept and Non groups were not present in the parental species, so they may be novel chemicals in the hybrids. The ASs of hybrids synthesizing mostly octanoic acid can be tested for insecticidal activity.

"Tinkerbell", "Baby Bella" and "Whisper"

The ornamental hybrids "Tinkerbell" (*Nicotiana alata* "Tinkerbell"), *Nicotiana* × hybrida "Baby Bella" (*Nicotiana* × *sanderae* "Baby Bella") and "Whisper" are highly disease-tolerant hybrids that have a notable pleasant aroma and attractive petal colors (https://www.gardenersworld.com/plants/nicotiana-alata-tinkerbell/; https://www.robsplants.com/plants/NicotSande; https://floranova.com/products/Nicotiana/; https://portlandnursery.com/annuals/nicotiana/). These hybrids possess the most diverse AS acyl profiles compared to the other studied hybrids. Seventeen acyl groups were detected (Table 7). The hybrids' AS acyl composition was qualitatively similar, consisting mostly of Oct, and 2-MeBut (40–50% of total acyl groups) along with numerous but minor (< 10% each) straight-chain acids ($C_{3,\ 4,\ 6–12}$) and branched-chain acids (MePro, 3-MeBut, MeVal, 4-MeHex and MeHept isomers).The acyl composition was similar to that of *N. alata*, and *N.* × *sanderae*, but it was enriched with six additional groups (Table 7). Hept, Non, Dec and Laur acyl groups may be novel for all the hybrids. There was variability in the relative proportions of AS acids among individual plants and among the hybrids. Oct was more prominent in "Baby Bella", while in "Tinkerbell" 2-MeBut was a predominant group followed by Oct and Ac. In "Whisper" the proportions between Oct and 2-MeBut varied from plant to plant (Table 7). MeHex isomers were found in the "Whisper" plants, but not in "Thinkerbell". In "Baby Bella" MePro varied from 0.5 to 13.8%. Oct varied from 7.2 to 34.5% in "Thinkerbell", and from 12 to 45% in "Wispers" (Table 7). Regarding the abundance of major acyl groups, the acyl compositions of "Tinkerbell" was more similar to *N. alata*, while "Baby Bella" acyl composition was more similar to *N.* × *sanderae*.

Table 7 AS acyl composition of horticulture hybrids "Thinkerbell", "Baby Bella" and "Whisper"

Acyl group	*N. alata*[1]	*N.* × *sanderae*[1]	"Thinkerbell"			"Baby Bella"			"Whisper"		
			1	2	3	1	2	3	1	2	3
			mol% of total								
Ac	23.9	21.5	32.0	–	13.0	21.5	–	4.4	3.3	3.0	1.4
Pro	–	–	5.4	–	3.7	5.3	–	–	-	-	-
MePro	14.3	3.8	13.3	3.1	12.2	13.8	0.5	4.7	6.3	5.5	1.5
But	7.4	3.6	0.9	1.5	2.0	9.7	1.1	4.4	3.7	2.5	1.0
2-MeBut	20.4	30.2	32.9	38.4	37.3	–	14.7	22.0	23.8	20.8	21.3
3-MeBut	6.9	0.9	2.7	2.3	3.2	–	1.3	2.6	3.8	3.5	3.2
3-MeVal	3.9	1.9	1.2	3.6	4.8	6.8	0.7	–	6.2	4.6	2.1
4-MeVal	–	–	tr	1.2	tr	0.7	0.8	–	3.0	2.2	0.6
Hex	6.2	3.5	0.8	2.6	1.5	4.6	6.3	3.1	3.5	2.9	3.0
4-MeHex	–	–	0.5	–	0.3	–	–	–	5.6	3.4	1.0
5-MeHex			0.4	–	0.3	–	–	–	–	–	0.8
Hept	–	–	0.3	2.0	0.7	1.7	4.5	1.9	4.1	2.7	1.7
5-MeHept	–	–	–	–	–	–	0.4	–	4.2	2.4	1.5
6-MeHept	–	–	–	–	–	–	0.5	–	3.5	2.7	1.5
Oct	16.8	34.7	7.2	34.5	16.3	31.2	57.9	37.8	12.7	30.8	45.1
Non	–	–	1.0	5.7	2.2	3.5	9.6	5.0	4.2	3.8	5.0
Dec	–	–	1.1	4.0	2.1	0.9	1.3	13.1	8.0	5.8	7.1
Laur	–	–	0.3	1.0	0.4	0.3	0.4	0.9	3.9	3.3	2.9

N. alata and *N.* × *sanderae* are some of the parental species (on the basis of the commercial providers' information https://www.gardenersworld.com/plants/nicotiana-alata-tinkerbell/; https://www.robsplants.com/plants/NicotSande). The data for *N. alata* and *N.* × *sanderae* are averaged from three plants. Individual F1 plants from each hybrid are shown. The underlined acyl groups are the most abundant. The numbers represent mol% of total

– not observed

[1]Kroumova et al. (2016)

Discussion

In this work, we studied the inheritance of the trichome-produced AS acyl groups in interspecific hybrids in the genus *Nicotiana* for the purpose of finding unique acyl compositions, combining diverse acyl groups from both parents. Of great interest was the inheritance of medium-length, straight or branched-chain groups as major groups in the ASs because they have been shown to be more toxic to arthropod pests (Escobar-Bravo et al. 2016; Jackson and Danehower 1996; Jackson et al. 1998; Severson et al. 1991; Simonovska et al. 2006; Song et al. 2006).

The parental species of *Nicotiana* hybrids have diverse acyl profiles. The combined genomes in the hybrids would carry all the genes necessary for synthesis of ASs and AS acyl groups featured in both parents and the interaction between the alleles from both genomes will determine the final profile in the hybrids. There is not sufficient knowledge about the genes and the regulatory factors participating in the synthesis of ASs in the genus *Nicotiana* to satisfactorily explain the observed patterns of inheritance. Figure S3 shows suggested α-KAE pathways for the synthesis of acyl-CoA groups (Kroumova et al. 1994) in some species of genus *Nicotiana* and in petunia. The pathways for the acyl groups' synthesis were concluded for *N. glutinosa*, *N. gossii*, and *N. bentamiana* (Kroumova and Wagner 2003). Numerous acyltransferase genes that play roles in acyl sugar assembly have been discovered among the species of genus *Solanum* and in petunia (Moghe et al. 2017; Nadakuduti et al. 2017) but orthologous acyltransferase genes have not been reported in *Nicotiana* species. We hypothesize that IPMS enzyme(s) (reaction 1, Fig. S3), branched-chain α-keto acid dehydrogenase (BCKD) enzyme(s) (reaction 3, Fig. S3), and putative acyl-transferases (reaction 4, Fig. S3) play roles in determination of the uniqueness of acyl groups in individual species, and for the variability in acyl sugar compositions commonly observed. Trichome-specific IPMS (reaction 1, Fig. S3) from *N. tabacum* has been characterized and was shown to be essential for the synthesis of 3-MeVal (Gwynn et al. 1985; https://www.lens.org/lens/patent/EP_2565265_A1), while the tomato E1-β subunit of BCKD (reaction 3, Fig. S3) was shown to affect branched-chain acyl groups in *N. benthamiana* (Slocombe et al. 2008). It is clear however, that many more genes are potentially relevant to AS acyl chain synthesis and ASs assembly, keeping in mind the great variability of ASs in the numerous *Nicotiana* species, and taking into consideration the voluminous work on the synthesis of ASs in the genus *Solanum* in recent years (Fan et al. 2016; Ning et al. 2015; Schilmiller et al. 2015, 2016). Genes involved in the metabolism of ASs have been studied extensively in solanaceous crops because of their important role in plant defense and also for the purpose of increasing AS-mediated pest resistance (Glas et al. 2012; Mandal et al. 2018; Moghe et al. 2017). Acyl sugar chemistry modification has been achieved in tomato by introducing individually, or in combination, several quantitative trait loci (QTLs) from *S. pennellii*. The introduction of more than one trait altered the abundance of total SE, the acyl group profile, and the diversity of SE due to the additive and epistatic interactions between the QTLs featuring certain genes (Leckie et al. 2012; Smeda et al. 2017).

Regardless of the potential participation of numerous genes in the synthesis of acyl groups and ASs assembly, it has been proposed that in the hybrids, in general, one or few genes exhibiting dominance/recessivity regulate the expression of secondary compounds (Gwynn et al. 1985; Lopes-Caamal and Tovar-Sanches 2014). Inheritance of some of the acyl groups in *Nicotiana* hybrids fits such a proposition, and is discussed below.

We have observed several patterns of AS acyl group inheritance (chemistry resembling that of a single parent, complementary acyl groups, missing acyl groups, and novel groups) that matched the patterns described by others for a variety of secondary metabolites, including essential oils, phenolic glucosides, cembratriene diols (CBT-diols), alkaloids, fatty acids, and phenolic constituents (Cheng et al. 2011; Court et al. 1992; Orians 2000). In addition, we observed variability in the expression of individual acyl groups. According to the literature, the first-generation hybrids (F_1) usually express both parental secondary metabolites (complementary expression) (Cheng et al. 2011; Rieseberg et al. Ellstrand 1993; Orians 2000), which is why our expectations were for the hybrid ASs to combine a variety of acyl groups in substantial abundance. In this study, complementary inheritance was observed but the expected abundance was not. In hybrids 110 and 114, all acyl groups were present, but Ac and MePro were not synthesized in the abundance that corresponded to that of the parental species. The overall acyl group profile of H110 and H114 resembled that of *N. benthamiana* (paternal inheritance) (Table 3). Similarly, it has been shown that in some *Nicotiana* hybrids the flower color was sometimes closer to one of the parents (Ling et al. 2012; Patel et al. 2011; Pental et al. 1984). When dark or flue-cured tobacco was crossed with *N. otophora*, the F_1 hybrids (H75 and H87) also had the acyl profile of both parents. However, the ratio of acyl groups and the major acyl groups did not resemble the profile of either parent (Table 4) but were similar to that of the oriental tobacco. We suggest that the strong reduction in 4-MeHex and the robust synthesis of 3-MeVal (already present in low abundance in the parents) in the hybrids may be caused by an isoform of IPMS (reaction 1, Fig. S3). Both acyl groups are products of the ante-iso branch of the α-ketoacid elongation pathway (α-KAE) (Fig. S3), where one cycle of elongation produces

3-MeVal, and two cycles produce 4-MeHex (Kroumova and Wagner 2003). The IPMS gene for synthesis of 4-MeHex in *N. otophora* may be in a heterozygous state, in which the dominant allele was not inherited in both hybrids, thus leading to the accumulation of the precursor 2-oxo-4 MeHex for synthesis of 3-MeVal.

The 5-and 6-MeHept groups produced by *N. benthamiana* were also inherited in *N. excelsiana* (Table 5). We hypothesize that in *N. benthamiana* both alleles of the putative gene(s) responsible for synthesis of MeHept isomers are in a dominant state, causing synthesis to occur in the hybrid. We think that one candidate gene important for the presence of MeHept in ASs is BCKD that is involved in branched-chain amino acid degradation and is potentially relevant to AS branched-chain acid synthesis (Slocombe et al. 2008). After BCKD E1-β silencing (reaction 3, Fig. S3) in *N. benthamiana*, a significant reduction in 5- and 6-MeHept was observed, while in *S. pennellii,* reduction was observed in the synthesis of MePro and 8-MeNon (Slocombe et al. 2008). Acyltransferase genes can be involved as well. Acyltransferases were shown to contribute to acylsugar diversity in the genus *Solanum* (Schilmiller et al. 2015). We suggest that in the genus *Nicotiana*, genes orthologous to those in *Solanum* may participate in the assembly of acylsugars.

In hybrid H37 (*N. excelsior* × *N. bigelovii*), Hept and Oct were not inherited, while 6-MeHept became a second major group in contrast to both parents where it had a minor presence. MeHex isomers were also elevated compared to their minimal presence in both parents (and 6-MeHept). We suggest that in this particular hybrid, ASAT enzymes may be involved (reaction 4, Fig. S3).

The Oct group from *N. alata* was inherited in the hybrids regardless of the SE acyl composition of the other parent. We hypothesize that, similar to MeHept in *N. benthamia*, the gene(s) important for Oct synthesis is in a homozygous dominant state and the inheritance is manifest in the hybrids. In contrast, Oct and Hept from *N. excelsior*, and 4-and 5-MeHex from *N. forgetiana* or *N. otophora*, were not inherited. It is possible that the genes involved in the synthesis of these groups are in a heterozygous state, and that the hybrids inherited the recessive allele (or the allele that is not functional). Acetate was not inherited in the *Alatae* hybrids. Most likely this may be related to acetyl-specific acyltransferase.

The appearance of novel acyl groups was observed, interestingly, only in *Alatae* hybrids and in the horticulture hybrids. The horticulture hybrids are also related to *Alatae* parents, and we may suggest that the novel groups are features in *Alatae* hybrids. Orians (2000) indicated that novelty may be more common for plants that produce chemicals in specialized cells, i.e. glandular trichomes. These novel acyl groups (together with Hex and Oct that are present in the parental species) may have come from the straight-chain branch of the α-KAE pathway (Fig. S3) as in petunia and *N. gossei* (Kroumova and Wagner 2003). It is also possible to be synthesized by fatty acid elongation path, via two-carbon elongation, characteristic of fatty acid biosynthesis (Kroumova and Wagner 2003; Walters and Steffens 1990).

The data presented in this paper show variability in acyl group inheritance in *Nicotiana* F_1 hybrids, and thus acyl profile prediction based on the chemotypes of the parental species could be inaccurate. Hybrids may inherit the acyl groups from both parents, but it is important also which acyl groups are in abundance and which groups will contribute to the pest resistance or will improve the hybrid fitness in the environment. In *N. tabacum* cv. Samsun-nn × *N. benthamiana* hybrids, 3-MeVal and MeBut isomers became minor groups. Similar changes were observed for Ac, MeBut isomers, and MeHex isomers in hybrids 75 and 87, while MeHept isomers were slightly increased (Table 4). Straight-chain groups from *N. excelsior* were not inherited. In contrast, in *N.* × *sanderae*, the straight-chain acyl groups Hex and Oct were inherited maternally from *N. alata*. The horticulture hybrids are said to be highly disease-tolerant (https://www.plant-world-seeds.com), and we assume that Oct contributes to this resistance. Synthetic sucrose octanoate esters have strong pesticidal and insecticidal effects (Michaud and McKenzie 2004; Technical Evaluation Report Sucrose Octanoate Esters 2005). The natural octanoate-containing ASs are synthesized in the species from section *Alatae* (Chortyk et al. 1993; Cutler et al. 1992; Kroumova et al. 2016, and current work). Thus, octanoic acid could be a good candidate for introgression into tobacco.

We did not find any correlation between the parental origins of individual acyl groups and their inheritance in hybrids. In *N. tabacum* cv. Samsun-nn, hybrids 110/114, and *N. excelsiana*, the inheritance of the major acyl groups was paternal. In *N.* × *sanderae* inheritance was maternal, while in H75 and H87 (*N. tabacum* × *N. otophora*) and in H37 the acyl profiles did not match either parent with respect to the major acyl groups.

This study shows different patterns of inheritance of AS acyl groups, and additional work is needed to further guide preparation of interspecific hybrids to provide sufficient amounts of unusual ASs in order to test their insect and disease resistance potential. Knowledge of the way that particular acyl groups function in plant protection could help increase trichome-based resistance in *N. tabacum*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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TOBACCO RESEARCH INCOME
INCOME COMPARISON

Fiscal Years	2012-2013	2013-2014	2014-2015	2015-2016	2016-2017	2017-2018	2018-2019	2019-2020
July	$ 23,478.65	$ (29,962.52)	$ 13,386.52	$ 139,619.47	$ 157,187.49	$ 2,459.48	$ 120,890.40	$ 141,864.01
August	$ 185,546.95	$ 343,968.71	$ 301,292.71	$ 177,916.68	$ 137,652.03	$ 292,266.42	$ 126,982.37	$ 145,789.42
September	$ 142,871.25	$ 152,700.22	$ 133,527.76	$ 47,768.58	$ 42,873.59	$ 139,414.92	$ 178,553.92	$ 132,169.60
1st QUARTER	**$ 351,896.85**	**$ 466,706.41**	**$ 448,206.99**	**$ 365,304.73**	**$ 337,713.11**	**$ 434,140.82**	**$ 426,426.69**	**$ 419,823.03**
October	$ 176,915.29	$ 148,858.82	$ 166,587.97	$ 255,006.22	$ 157,120.53	$ 126,862.91	$ 97,793.84	$ 150,849.00
November	$ 231,929.57	$ 55,176.52	$ 74,462.42	$ 127,495.52	$ 251,055.77	$ 123,267.74	$ 128,963.50	$ 117,280.34
December	$ 218,829.28	$ 136,860.86	$ 190,289.54	$ 26,196.02	$ 113,251.82	$ 135,314.04	$ 175,277.00	$ 151,323.23
2nd QUARTER	**$ 627,674.14**	**$ 340,896.20**	**$ 431,339.93**	**$ 408,697.76**	**$ 521,428.12**	**$ 385,444.69**	**$ 402,034.34**	**$ 419,452.57**
SIX MONTHS	**$ 979,570.99**	**$ 807,602.61**	**$ 879,546.92**	**$ 774,002.49**	**$ 859,141.23**	**$ 819,585.51**	**$ 828,461.03**	**$ 839,275.60**
January	$ 143,268.63	$ 258,621.49	$ 44,597.62	$ 264,622.53	$ 109,584.57	$ 127,719.90	$ 564,217.88	$ 120,247.87
February	$ 11,634.70	$ 115,623.41	$ 212,408.73	$ 10,472.72	$ 155,644.33	$ 114,047.53	$ 141,118.46	$ 114,095.14
March	$ 194,538.93	$ 57,626.04	$ 133,593.90	$ 255,769.54	$ 159,012.56	$ 159,645.83	$ 122,472.86	$ 403,962.17
3rd QUARTER	**$ 349,442.26**	**$ 431,870.94**	**$ 390,600.25**	**$ 530,864.79**	**$ 424,241.46**	**$ 401,413.26**	**$ 827,809.20**	**$ 638,305.18**
NINE MONTHS	**$ 1,329,013.25**	**$ 1,239,473.55**	**$ 1,270,147.17**	**$ 1,304,867.28**	**$ 1,283,382.69**	**$ 1,220,998.77**	**$ 1,656,270.23**	**$ 1,477,580.78**
April	$ 240,079.82	$ 243,424.06	$ 165,299.42	$ 20,461.50	$ 43,764.59	$ 65,036.15	$ 146,789.57	$ 117,862.64
May	$ 81,297.53	$ 24,607.31	$ 183,052.97	$ 144,713.80	$ 174,933.87	$ 209,087.27	$ 63,797.02	$ 141,525.18
June	$ 282,726.79	$ 296,886.53	$ 137,563.73	$ 288,160.23	$ 242,003.23	$ 168,621.20	$ 250,352.13	$ 138,849.18
4th QUARTER	**$ 604,104.14**	**$ 564,917.90**	**$ 485,916.12**	**$ 453,335.53**	**$ 460,701.69**	**$ 442,744.62**	**$ 460,938.72**	**$ 398,237.00**
TOTAL INCOME	**$ 1,933,117.39**	**$ 1,804,391.45**	**$ 1,756,063.29**	**$ 1,758,202.81**	**$ 1,744,084.38**	**$ 1,663,743.39**	**$ 2,117,208.95**	**$ 1,875,817.78**

Funds Center		Commitment item	Annual (Revised)Budget	PriorBalance	Current MonthActual	YTD Actual	Available Budget
1235410080	HOLDING ACCOUNT	All Budget Commitmen	$ (1,750,000.00)	$ (1,736,968.60)	$ (138,849.18)	$ (1,875,817.78)	$ 125,817.78
1235410080	HOLDING ACCOUNT	All Revenue Commitme	$ (1,750,000.00)	$ (1,736,968.60)	$ (138,849.18)	$ (1,875,817.78)	$ 125,817.78
1235410080	HOLDING ACCOUNT	TTL Revenue Excl Tra	$ (1,750,000.00)	$ (1,736,968.60)	$ (138,849.18)	$ (1,875,817.78)	$ 125,817.78
1235410080	HOLDING ACCOUNT	State/Local Grants a	$ (1,325,000.00)	$ (1,736,968.60)	$ (138,849.18)	$ (1,875,817.78)	$ 550,817.78
1235410080	HOLDING ACCOUNT	Fund Balance	$ (425,000.00)				$ (425,000.00)
1235410090	KENTUCKY TOBACCO RES	All Budget Commitmen	$ 1,000.00	$ 493.77		$ 493.77	$ 506.23
1235410090	KENTUCKY TOBACCO RES	All Expenses Commitm	$ 1,000.00	$ 493.77		$ 493.77	$ 506.23
1235410090	KENTUCKY TOBACCO RES	TTL Expense Excl Tra	$ 1,000.00	$ 493.77		$ 493.77	$ 506.23
1235410090	KENTUCKY TOBACCO RES	Operating Expense	$ 1,000.00	$ 493.77		$ 493.77	$ 506.23
1235410100	ADMINISTRATION	All Budget Commitmen	$ 230,628.00	$ 207,931.74	$ 21,272.14	$ 229,203.88	$ 1,367.74
1235410100	ADMINISTRATION	All Expenses Commitm	$ 230,628.00	$ 207,931.74	$ 21,272.14	$ 229,203.88	$ 1,367.74
1235410100	ADMINISTRATION	TTL Expense Excl Tra	$ 230,628.00	$ 207,931.74	$ 21,272.14	$ 229,203.88	$ 1,367.74
1235410100	ADMINISTRATION	Faculty		$ 73,562.96	$ 6,687.54	$ 80,250.50	$ (80,250.50)
1235410100	ADMINISTRATION	Staff		$ 72,145.91	$ 7,826.20	$ 79,972.11	$ (79,972.11)
1235410100	ADMINISTRATION	Other Personnel	$ 230,628.00	$ 101.75		$ 101.75	$ 230,526.25
1235410100	ADMINISTRATION	Fringe Benefits		$ 48,417.24	$ 5,104.03	$ 53,521.27	$ (53,521.27)
1235410100	ADMINISTRATION	Operating Expense		$ 13,703.88	$ 1,654.37	$ 15,358.25	$ (15,414.63)
1235410110	KTRDC PERSONNEL	All Budget Commitmen	$ 971,770.00	$ 592,506.10	$ 91,402.07	$ 683,908.17	$ 287,861.83
1235410110	KTRDC PERSONNEL	All Expenses Commitm	$ 971,770.00	$ 592,506.10	$ 91,402.07	$ 683,908.17	$ 287,861.83
1235410110	KTRDC PERSONNEL	TTL Expense Excl Tra	$ 971,770.00	$ 592,506.10	$ 91,402.07	$ 683,908.17	$ 287,861.83
1235410110	KTRDC PERSONNEL	Staff		$ 374,746.79	$ 60,539.53	$ 435,286.32	$ (435,286.32)
1235410110	KTRDC PERSONNEL	Other Personnel		$ 81,395.45	$ 8,555.59	$ 89,951.04	$ (89,951.04)
1235410110	KTRDC PERSONNEL	Fringe Benefits		$ 124,265.86	$ 21,083.75	$ 145,349.61	$ (145,349.61)
1235410110	KTRDC PERSONNEL	Operating Expense	$ 971,770.00	$ 12,098.00	$ 1,223.20	$ 13,321.20	$ 958,448.80

Funds Center		Commitment item	Annual (Revised)Budget	PriorBalance	Current MonthActual	YTD Actual	Available Budget
1235410120	PUBLICATIONS & TRAVE	All Budget Commitmen	$ 24,000.00	$ 3,951.82	$ 582.22	$ 4,534.04	$ 19,465.96
1235410120	PUBLICATIONS & TRAVE	All Expenses Commitm	$ 24,000.00	$ 3,951.82	$ 582.22	$ 4,534.04	$ 19,465.96
1235410120	PUBLICATIONS & TRAVE	TTL Expense Excl Tra	$ 24,000.00	$ 3,951.82	$ 582.22	$ 4,534.04	$ 19,465.96
1235410120	PUBLICATIONS & TRAVE	Operating Expense	$ 24,000.00	$ 3,951.82	$ 582.22	$ 4,534.04	$ 19,465.96
1235410130	BUILDING MAINTENANCE	All Budget Commitmen	$ 63,000.00	$ 47,755.61	$ 3,383.35	$ 51,138.96	$ 11,861.04
1235410130	BUILDING MAINTENANCE	All Expenses Commitm	$ 63,000.00	$ 47,755.61	$ 3,383.35	$ 51,138.96	$ 11,861.04
1235410130	BUILDING MAINTENANCE	TTL Expense Excl Tra	$ 63,000.00	$ 47,755.61	$ 3,383.35	$ 51,138.96	$ 11,861.04
1235410130	BUILDING MAINTENANCE	Operating Expense	$ 63,000.00	$ 47,755.61	$ 3,383.35	$ 51,138.96	$ 11,861.04
1235410140	KTRDC PRO CARD	All Budget Commitmen		$ 3,618.78	$ (3,618.78)	$ -	$ -
1235410140	KTRDC PRO CARD	All Expenses Commitm		$ 3,618.78	$ (3,618.78)	$ -	$ -
1235410140	KTRDC PRO CARD	TTL Expense Excl Tra		$ 3,618.78	$ (3,618.78)	$ -	$ -
1235410140	KTRDC PRO CARD	Operating Expense		$ 3,618.78	$ (3,618.78)	$ -	$ -
1235410180	SHOP	All Budget Commitmen	$ 1,000.00	$ 144.82	$ 335.94	$ 480.76	$ 519.24
1235410180	SHOP	All Expenses Commitm	$ 1,000.00	$ 144.82	$ 335.94	$ 480.76	$ 519.24
1235410180	SHOP	TTL Expense Excl Tra	$ 1,000.00	$ 144.82	$ 335.94	$ 480.76	$ 519.24
1235410180	SHOP	Operating Expense	$ 1,000.00	$ 144.82	$ 335.94	$ 480.76	$ 519.24
1235410240	LABORATORY EQUIPMENT	All Budget Commitmen	$ 20,000.00	$ 8,032.82		$ 8,032.82	$ 11,967.18
1235410240	LABORATORY EQUIPMENT	All Expenses Commitm	$ 20,000.00	$ 8,032.82		$ 8,032.82	$ 11,967.18
1235410240	LABORATORY EQUIPMENT	TTL Expense Excl Tra	$ 20,000.00	$ 8,032.82		$ 8,032.82	$ 11,967.18
1235410240	LABORATORY EQUIPMENT	Operating Expense	$ 20,000.00	$ 8,032.82		$ 8,032.82	$ 11,967.18
1235410250	UNALLOCATED RESERVE	All Budget Commitmen	$ 154,602.00				$ 154,602.00
1235410250	UNALLOCATED RESERVE	All Expenses Commitm	$ 154,602.00				$ 154,602.00
1235410250	UNALLOCATED RESERVE	TTL Expense Excl Tra	$ 154,602.00				$ 154,602.00
1235410250	UNALLOCATED RESERVE	Operating Expense	$ 154,602.00				$ 154,602.00

Funds Center		Commitment item	Annual (Revised)Budget	PriorBalance	Current MonthActual	YTD Actual	Available Budget
1235410280	GENERAL LABORATORY	All Budget Commitmen	$ 65,000.00	$ 40,671.19	$ 2,441.21	$ 43,112.40	$ 21,635.50
1235410280	GENERAL LABORATORY	All Expenses Commitm	$ 65,000.00	$ 40,671.19	$ 2,441.21	$ 43,112.40	$ 21,635.50
1235410280	GENERAL LABORATORY	TTL Expense Excl Tra	$ 65,000.00	$ 40,671.19	$ 2,441.21	$ 43,112.40	$ 21,635.50
1235410280	GENERAL LABORATORY	Operating Expense	$ 65,000.00	$ 40,671.19	$ 2,441.21	$ 43,112.40	$ 21,635.50
1235411040	DISCRETIONARY	All Budget Commitmen	$ 9,000.00	$ 7,308.38	$ (76.48)	$ 7,231.90	$ 1,768.10
1235411040	DISCRETIONARY	All Revenue Commitme			$ 0.53	$ 0.53	$ (0.53)
1235411040	DISCRETIONARY	Revenue Transfers			$ 0.53	$ 0.53	$ (0.53)
1235411040	DISCRETIONARY	Trans fm UK Restric			$ 0.53	$ 0.53	$ (0.53)
1235411040	DISCRETIONARY	All Expenses Commitm	$ 9,000.00	$ 7,308.38	$ (77.01)	$ 7,231.37	$ 1,768.63
1235411040	DISCRETIONARY	TTL Expense Excl Tra	$ 9,000.00	$ 7,308.38	$ (77.01)	$ 7,231.37	$ 1,768.63
1235411040	DISCRETIONARY	Operating Expense	$ 9,000.00	$ 7,308.38	$ (77.01)	$ 7,231.37	$ 1,768.63
1235411320	PLANT GENETIC ENGR	All Budget Commitmen	$ 30,000.00	$ 30,043.40		$ 30,043.40	$ (43.40)
1235411320	PLANT GENETIC ENGR	All Expenses Commitm	$ 30,000.00	$ 30,043.40		$ 30,043.40	$ (43.40)
1235411320	PLANT GENETIC ENGR	TTL Expense Excl Tra	$ 30,000.00	$ 30,043.40		$ 30,043.40	$ (43.40)
1235411320	PLANT GENETIC ENGR	Staff		$ 20,426.48		$ 20,426.48	$ (20,426.48)
1235411320	PLANT GENETIC ENGR	Other Personnel		$ 782.76		$ 782.76	$ (782.76)
1235411320	PLANT GENETIC ENGR	Fringe Benefits		$ 7,549.75		$ 7,549.75	$ (7,549.75)
1235411320	PLANT GENETIC ENGR	Operating Expense	$ 30,000.00	$ 1,284.41		$ 1,284.41	$ 28,715.59
1235411340	GENETIC MANIPULATION	All Budget Commitmen	$ 30,000.00	$ 30,480.01		$ 30,480.01	$ (480.01)
1235411340	GENETIC MANIPULATION	All Expenses Commitm	$ 30,000.00	$ 30,480.01		$ 30,480.01	$ (480.01)
1235411340	GENETIC MANIPULATION	TTL Expense Excl Tra	$ 30,000.00	$ 30,480.01		$ 30,480.01	$ (480.01)
1235411340	GENETIC MANIPULATION	Staff		$ 16,897.92		$ 16,897.92	$ (16,897.92)
1235411340	GENETIC MANIPULATION	Other Personnel		$ 5,370.23		$ 5,370.23	$ (5,370.23)
1235411340	GENETIC MANIPULATION	Fringe Benefits		$ 4,978.59		$ 4,978.59	$ (4,978.59)
1235411340	GENETIC MANIPULATION	Operating Expense	$ 30,000.00	$ 3,233.27		$ 3,233.27	$ 26,766.73

Funds Center		Commitment item	Annual (Revised)Budget	PriorBalance	Current MonthActual	YTD Actual	Available Budget
1235411370	PLANT BIOTECH MOLECU	All Budget Commitmen	$ 30,000.00	$ 14,578.89	$ 5,645.72	$ 20,224.61	$ 9,775.39
1235411370	PLANT BIOTECH MOLECU	All Expenses Commitm	$ 30,000.00	$ 14,578.89	$ 5,645.72	$ 20,224.61	$ 9,775.39
1235411370	PLANT BIOTECH MOLECU	TTL Expense Excl Tra	$ 30,000.00	$ 14,578.89	$ 5,645.72	$ 20,224.61	$ 9,775.39
1235411370	PLANT BIOTECH MOLECU	Other Personnel		$ 2,023.90		$ 2,023.90	$ (2,023.90)
1235411370	PLANT BIOTECH MOLECU	Fringe Benefits		$ 28.54		$ 28.54	$ (28.54)
1235411370	PLANT BIOTECH MOLECU	Operating Expense	$ 30,000.00	$ 12,526.45	$ 5,645.72	$ 18,172.17	$ 11,827.83
1235411380	MOLECULAR GENETICS	All Budget Commitmen	$ 30,000.00	$ 6,921.36	$ 9,874.89	$ 16,796.25	$ 13,203.75
1235411380	MOLECULAR GENETICS	All Expenses Commitm	$ 30,000.00	$ 6,921.36	$ 9,874.89	$ 16,796.25	$ 13,203.75
1235411380	MOLECULAR GENETICS	TTL Expense Excl Tra	$ 30,000.00	$ 6,921.36	$ 9,874.89	$ 16,796.25	$ 13,203.75
1235411380	MOLECULAR GENETICS	Other Personnel		$ 3,051.92	$ 3,051.92	$ 6,103.84	$ (6,103.84)
1235411380	MOLECULAR GENETICS	Fringe Benefits		$ 663.40	$ 663.40	$ 1,326.80	$ (1,326.80)
1235411380	MOLECULAR GENETICS	Operating Expense	$ 30,000.00	$ 3,206.04	$ 6,159.57	$ 9,365.61	$ 20,634.39
1235411410	GREENHOUSE	All Budget Commitmen	$ 30,000.00	$ 11,938.56	$ 4,277.48	$ 16,216.04	$ 13,748.99
1235411410	GREENHOUSE	All Expenses Commitm	$ 30,000.00	$ 11,938.56	$ 4,277.48	$ 16,216.04	$ 13,748.99
1235411410	GREENHOUSE	TTL Expense Excl Tra	$ 30,000.00	$ 11,938.56	$ 4,277.48	$ 16,216.04	$ 13,748.99
1235411410	GREENHOUSE	Other Personnel			$ 3,000.72	$ 3,000.72	$ (3,000.72)
1235411410	GREENHOUSE	Fringe Benefits		$ (0.47)	$ 164.08	$ 163.61	$ (163.61)
1235411410	GREENHOUSE	Operating Expense	$ 30,000.00	$ 11,939.03	$ 1,112.68	$ 13,051.71	$ 16,913.32
1235411640	GENE DISCOVERY	All Budget Commitmen	$ 30,000.00	$ 33,529.99	$ (3,324.09)	$ 30,205.90	$ (205.90)
1235411640	GENE DISCOVERY	All Expenses Commitm	$ 30,000.00	$ 33,529.99	$ (3,324.09)	$ 30,205.90	$ (205.90)
1235411640	GENE DISCOVERY	TTL Expense Excl Tra	$ 30,000.00	$ 33,529.99	$ (3,324.09)	$ 30,205.90	$ (205.90)
1235411640	GENE DISCOVERY	Staff		$ 22,641.98	$ (2,515.78)	$ 20,126.20	$ (20,126.20)
1235411640	GENE DISCOVERY	Fringe Benefits		$ 8,075.54	$ (897.29)	$ 7,178.25	$ (7,178.25)
1235411640	GENE DISCOVERY	Operating Expense	$ 30,000.00	$ 2,812.47	$ 88.98	$ 2,901.45	$ 27,098.55

Funds Center		Commitment item	Annual (Revised)Budget	PriorBalance	Current MonthActual	YTD Actual	Available Budget
1235411750	REFERENCE CIGARETTES	All Budget Commitmen			$ 82,098.93	$ 82,098.93	$ (82,098.93)
1235411750	REFERENCE CIGARETTES	All Expenses Commitm			$ 82,098.93	$ 82,098.93	$ (82,098.93)
1235411750	REFERENCE CIGARETTES	TTL Expense Excl Tra			$ 82,098.93	$ 82,098.93	$ (82,098.93)
1235411750	REFERENCE CIGARETTES	Operating Expense			$ 82,098.93	$ 82,098.93	$ (82,098.93)
1235412240	GREENHOUSE EVALUATIO	All Budget Commitmen			$ 1,453.17	$ 1,453.17	$ (1,453.17)
1235412240	GREENHOUSE EVALUATIO	All Expenses Commitm			$ 1,453.17	$ 1,453.17	$ (1,453.17)
1235412240	GREENHOUSE EVALUATIO	TTL Expense Excl Tra			$ 1,453.17	$ 1,453.17	$ (1,453.17)
1235412240	GREENHOUSE EVALUATIO	Other Personnel			$ 1,332.45	$ 1,332.45	$ (1,332.45)
1235412240	GREENHOUSE EVALUATIO	Fringe Benefits			$ 120.72	$ 120.72	$ (120.72)
1235412360	FLAVONOID - SMALLE	All Budget Commitmen	$ 30,000.00	$ 10,897.92	$ 6,365.25	$ 17,263.17	$ 12,287.16
1235412360	FLAVONOID - SMALLE	All Expenses Commitm	$ 30,000.00	$ 10,897.92	$ 6,365.25	$ 17,263.17	$ 12,287.16
1235412360	FLAVONOID - SMALLE	TTL Expense Excl Tra	$ 30,000.00	$ 10,897.92	$ 6,365.25	$ 17,263.17	$ 12,287.16
1235412360	FLAVONOID - SMALLE	Staff		$ 3,321.69	$ 4,877.59	$ 8,199.28	$ (8,199.28)
1235412360	FLAVONOID - SMALLE	Fringe Benefits		$ 1,258.97	$ 1,487.66	$ 2,746.63	$ (2,746.63)
1235412360	FLAVONOID - SMALLE	Operating Expense	$ 30,000.00	$ 6,317.26		$ 6,317.26	$ 23,233.07
1235412790	JI: PURIFICATION OF	All Budget Commitmen			$ 623.54	$ 623.54	$ (623.54)
1235412790	JI: PURIFICATION OF	All Expenses Commitm			$ 623.54	$ 623.54	$ (623.54)
1235412790	JI: PURIFICATION OF	TTL Expense Excl Tra			$ 623.54	$ 623.54	$ (623.54)
1235412790	JI: PURIFICATION OF	Operating Expense			$ 623.54	$ 623.54	$ (623.54)
1235412840	MARTINEZ: GREENHOUSE	All Budget Commitmen			$ 828.89	$ 828.89	$ (828.89)
1235412840	MARTINEZ: GREENHOUSE	All Expenses Commitm			$ 828.89	$ 828.89	$ (828.89)
1235412840	MARTINEZ: GREENHOUSE	TTL Expense Excl Tra			$ 828.89	$ 828.89	$ (828.89)
1235412840	MARTINEZ: GREENHOUSE	Operating Expense			$ 828.89	$ 828.89	$ (828.89)

Funds Center		Commitment item	Annual (Revised)Budget	PriorBalance	Current MonthActual	YTD Actual	Available Budget
1235413080	PERR INCREASED TAG	All Budget Commitmen		$ 51.29		$ 51.29	$ (51.29)
1235413080	PERR INCREASED TAG	All Expenses Commitm		$ 51.29		$ 51.29	$ (51.29)
1235413080	PERR INCREASED TAG	TTL Expense Excl Tra		$ 51.29		$ 51.29	$ (51.29)
1235413080	PERR INCREASED TAG	Operating Expense		$ 51.29		$ 51.29	$ (51.29)
1235413200	PERRY: PROD MAL	All Budget Commitmen			$ 1,059.48	$ 1,059.48	$ (1,059.48)
1235413200	PERRY: PROD MAL	All Expenses Commitm			$ 1,059.48	$ 1,059.48	$ (1,059.48)
1235413200	PERRY: PROD MAL	TTL Expense Excl Tra			$ 1,059.48	$ 1,059.48	$ (1,059.48)
1235413200	PERRY: PROD MAL	Operating Expense			$ 1,059.48	$ 1,059.48	$ (1,059.48)
1235413220	SCHU: SEED SHATTERNG	All Budget Commitmen		$ 255.03		$ 255.03	$ (255.03)
1235413220	SCHU: SEED SHATTERNG	All Expenses Commitm		$ 255.03		$ 255.03	$ (255.03)
1235413220	SCHU: SEED SHATTERNG	TTL Expense Excl Tra		$ 255.03		$ 255.03	$ (255.03)
1235413220	SCHU: SEED SHATTERNG	Other Personnel		$ 260.00		$ 260.00	$ (260.00)
1235413220	SCHU: SEED SHATTERNG	Fringe Benefits		$ (4.97)		$ (4.97)	$ 4.97