

The enantiomeric composition of N'-nitrosonornicotine in air-cured tobacco influenced by nicotine demethylation

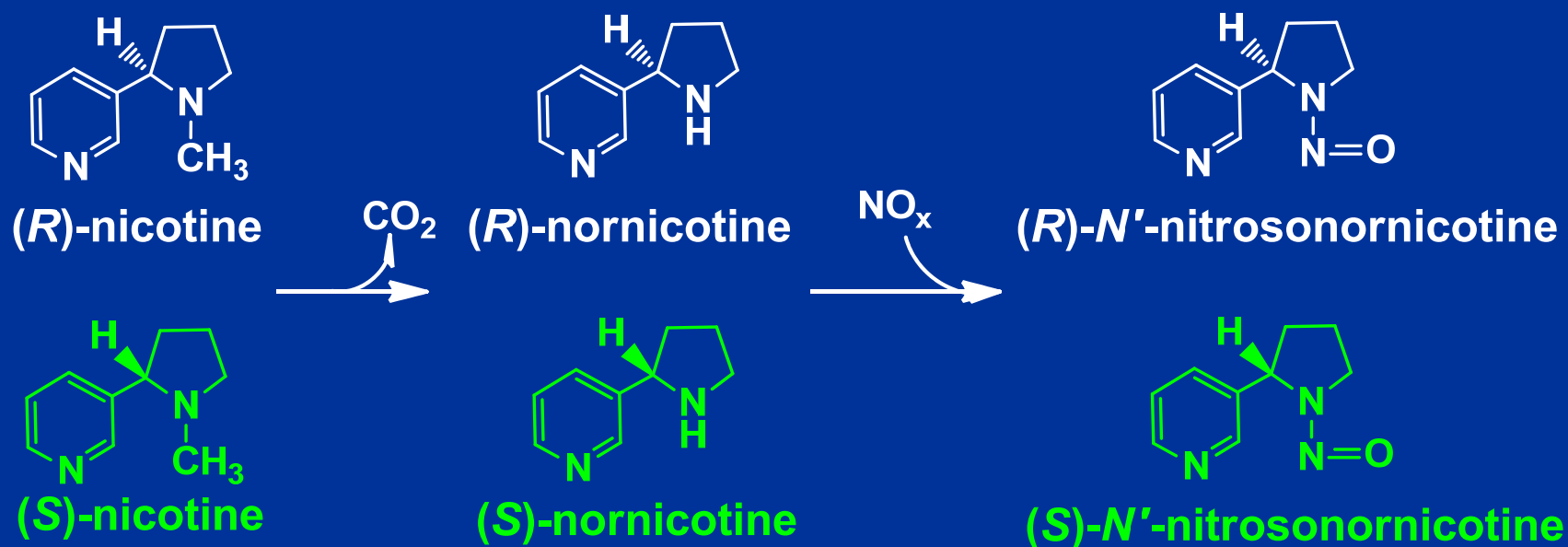
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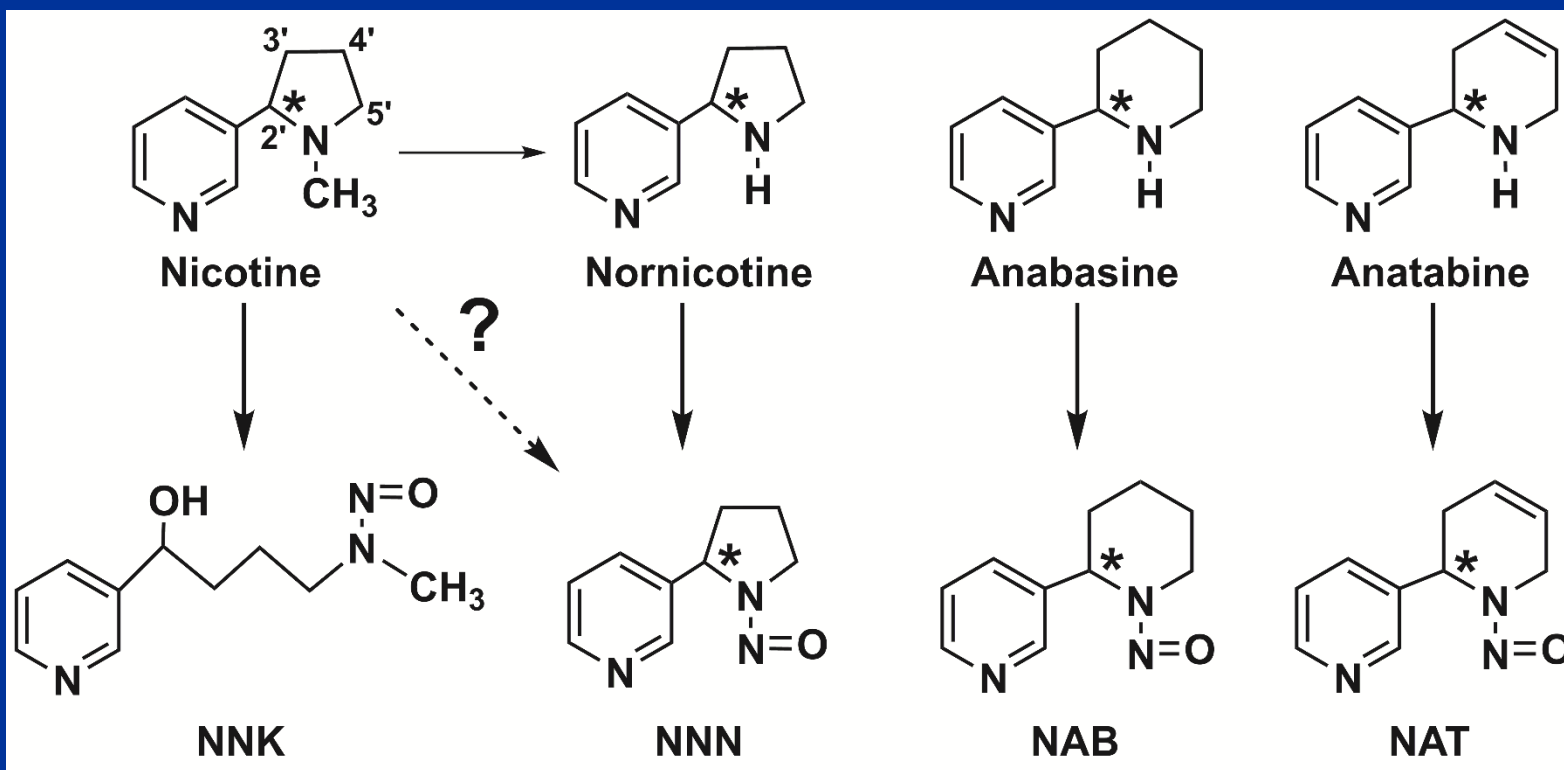
2017 TSRC

Alkaloids and TSNAs are all chiral compounds



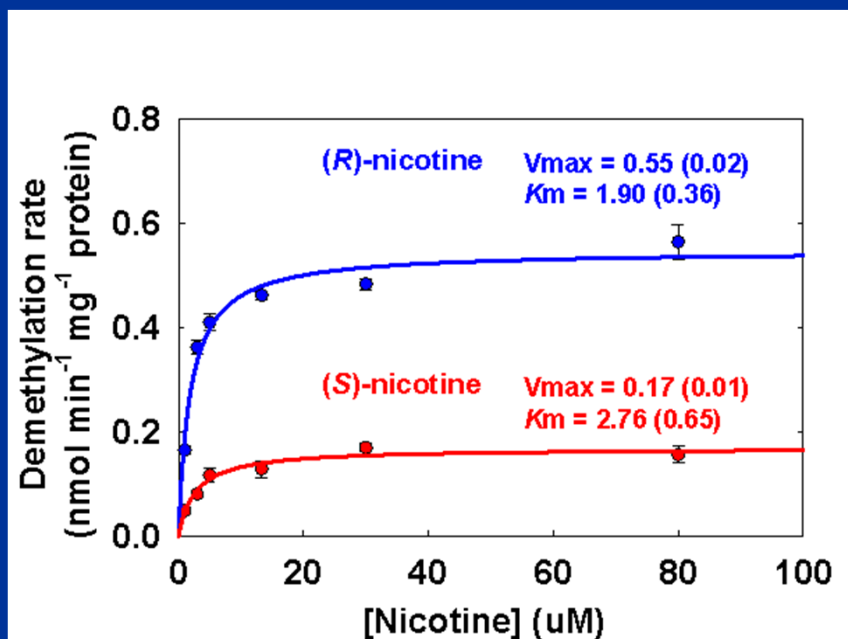
(S)-NNN is more carcinogenic than (R)-NNN.

Overview of the nitrosation reactions leading to the TSNAs formation in tobacco leaf

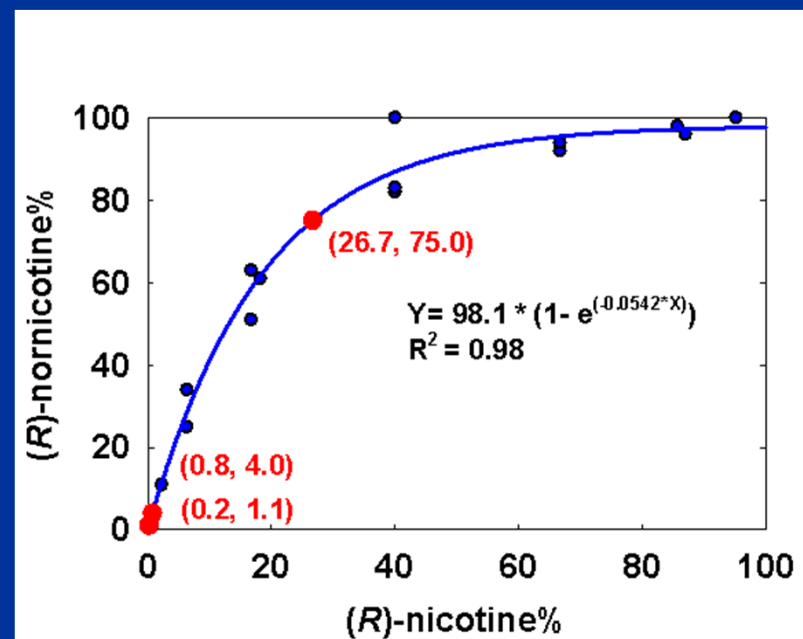


Three nicotine demethylases reported: CYP82E4(E4), CYP82E5(E5) and CYP82E10(E10).

In vitro kinetics study of E4 demethylation



E4 shows preference for (R)-nicotine *in vitro*.



E4 alone can not produce 4-75% (R)-nornicotine percentage from 0.2% (R)-nicotine.

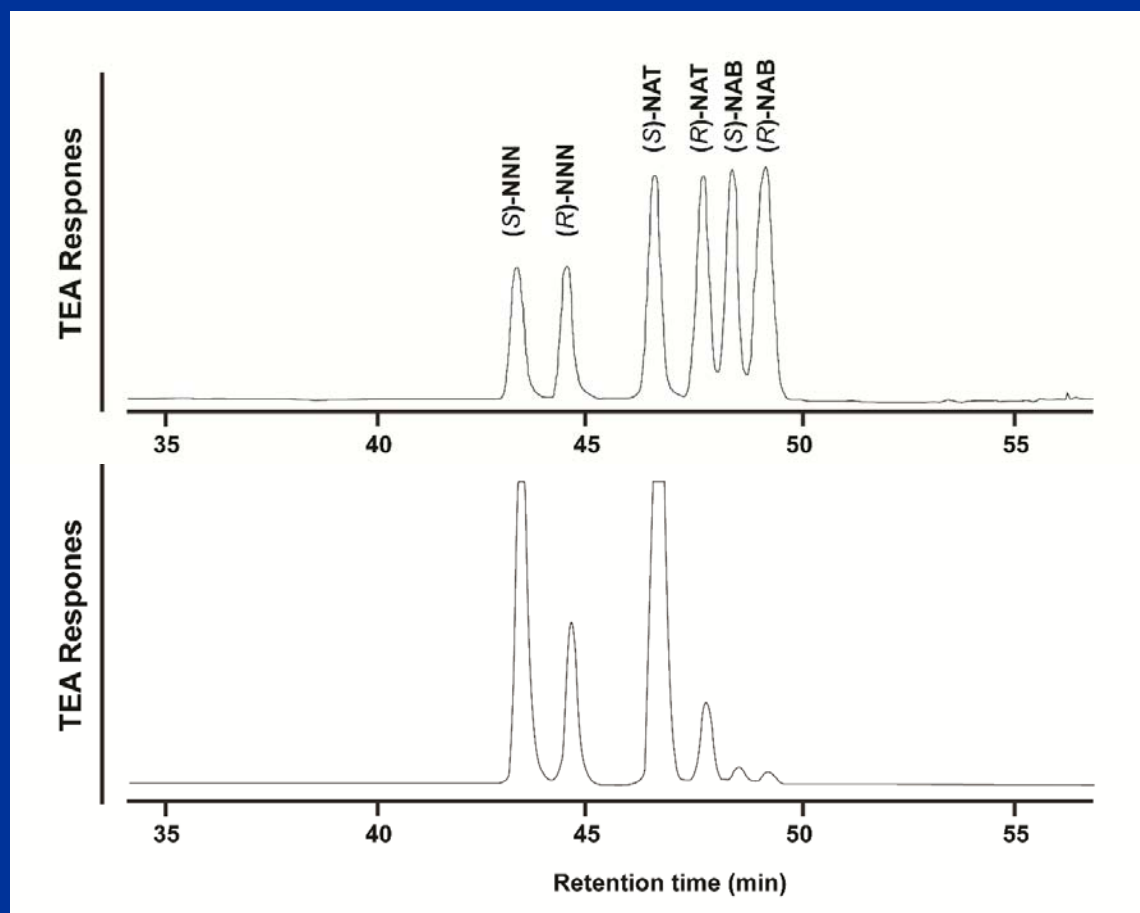
Questions

- ❖ what is the contribution of nicotine and nornicotine to the accumulation of NNN present in triple mutant line (*e4e5e10*) ?
- ❖ What are the effects of three demethylases on enantiomeric compositions of TSNAs?

Experimental design

- ❖ Tobacco lines with different combinations of three nicotine demethylases inhibited were grown in the field.
- ❖ Air-cured leaves were analyzed for the concentration and enantiomeric composition of nicotine, nornicotine and NNN.
- ❖ A gas chromatography/thermal energy analyzer method using two columns in series was developed to separate the enantiomers of NNN, N'-nitrosoanabasine, and N'-nitrosoanatabine.

NNN, NAT and NAB enantiomers were separated by a tandem column



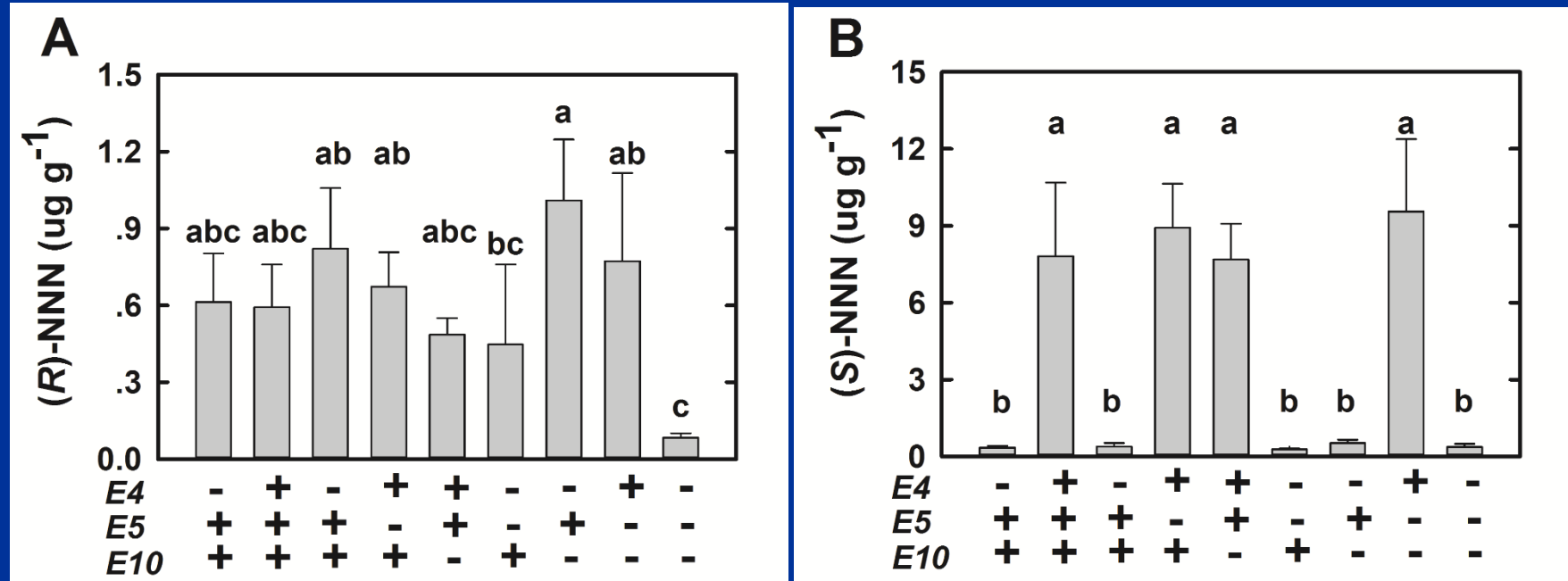
Gas chromatography of standard solution (mixtures of racemic NNN, NAT and NAB) and a TSNA extract from reference cigarette 2R1 tobacco

Enantiomeric compositions of nicotine, nornicotine, NNN, NAT and NAB in air-cured mutant lamina from 2011 field trial

	Treatment			R-nic%	R-nnic%	R-NNN%	R-NAT%	R-NAB%
	<i>E4</i>	<i>E5</i>	<i>E10</i>					
2R1				0.5	30.0	29.5	15.2	41.3
1R4F				0.3	29.0	26.6	16.5	40.5
TN 90LC	+ ^s	+	+	0.0 ± 0.0	66.7 ± 2.2	63.5 ± 2.6	15.5 ± 0.6	42.4
Parent	+	+	+	0.1 ± 0.1	6.0 ± 0.7	7.3 ± 1.3	17.3 ± 3.8	39.9
<i>e4E5E10</i>	-	+	+	0.0 ± 0.0	70.5 ± 3.5	69.0 ± 3.2	18.4 ± 2.7	41.7
<i>E4e5E10</i>	+	-	+	0.1 ± 0.0	6.5 ± 0.4	7.0 ± 0.2	15.6 ± 1.4	41.0
<i>E4E5e10</i>	+	-	-	0.4 ± 0.3	5.9 ± 0.2	6.0 ± 1.0	14.7 ± 1.2	36.3
<i>e4e5E10</i>	-	-	+	0.4 ± 0.0	65.2 ± 8.3	68.1 ± 0.4	17.1 ± 1.1	40.8
<i>e4E5e10</i>	-	+	-	0.1 ± 0.0	66.6 ± 3.6	66.5 ± 1.2	15.4 ± 0.4	44.8
<i>E4e5e10</i>	+	-	-	0.2 ± 0.1	8.0 ± 0.4	7.3 ± 1.1	13.5 ± 0.8	37.5
<i>e4e5e10</i>	-	-	-	3.8 ± 0.3	18.1 ± 2.5	19.3 ± 3.2	16.4 ± 0.9	40.8

(R)-NNN composition was almost the same as the (R)-nornicotine. NAT and NAB composition has not been affected by the mutation of nicotine demethylase genes

NNN enantiomer accumulation in air-cured mutant lamina from 2011 field trial

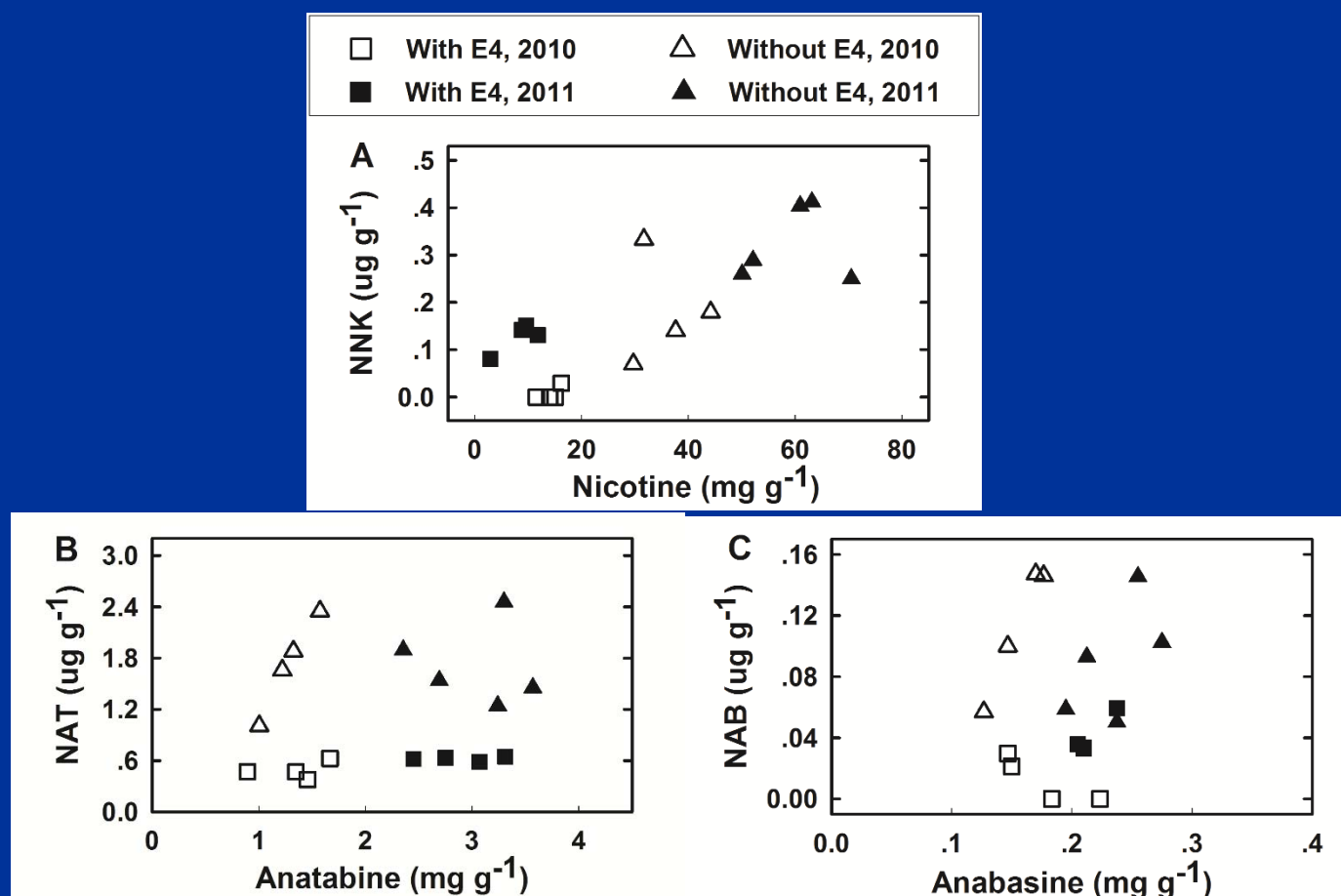


Three nicotine demethylases affect (*R*)-NNN accumulation, and CYP82E4 influences (*S*)-NNN accumulation.

Nicotine, nornicotine and NNN levels in air-cured lamina affected by different nicotine demethylase mutations

	Treatment			Nicotine mg g ⁻¹	Nornicotine mg g ⁻¹	NNN μg g ⁻¹	
	<i>E4</i>	<i>E5</i>	<i>E10</i>				
2010	<i>E4E5E10</i>	+	+	+	16.2 ± 3.5	24.80 ± 2.28	12.41 ± 3.75
	<i>e4E5E10</i>	-	+	+	29.7 ± 7.7	0.88 ± 0.22	1.16 ± 0.68
	<i>E4e5E10</i>	+	-	+	11.5 ± 1.7	20.86 ± 2.35	8.99 ± 3.42
	<i>E4E5e10</i>	+	-	-	15.0 ± 2.1	19.30 ± 4.00	10.18 ± 3.33
	<i>e4e5E10</i>	-	-	+	31.7 ± 2.5	0.90 ± 0.11	1.65 ± 0.50
	<i>e4E5e10</i>	-	+	-	37.6 ± 3.8	0.95 ± 0.11	1.59 ± 0.54
	<i>E4e5e10</i>	+	-	-	14.0 ± 0.5	18.34 ± 1.95	13.63 ± 3.91
	<i>e4e5e10</i>	-	-	-	44.2 ± 1.2	0.48 ± 0.04	0.75 ± 0.11
TN 90 LC ^a	+ ^s	+	+	70.5 ± 8.5	1.86 ± 0.12	0.96 ± 0.26	
2011	<i>E4E5E10</i>	+	+	+	11.8 ± 3.5	44.99 ± 8.20	8.40 ± 3.03
	<i>e4E5E10</i>	-	+	+	50.1 ± 5.2	1.63 ± 0.15	1.20 ± 0.38
	<i>E4e5E10</i>	+	-	+	8.8 ± 4.6	42.55 ± 2.68	9.60 ± 1.84
	<i>E4E5e10</i>	+	-	-	2.9 ± 0.9	43.10 ± 4.92	8.16 ± 1.42
	<i>e4e5E10</i>	-	-	+	52.1 ± 3.8	1.44 ± 0.21	0.72 ± 0.34
	<i>e4E5e10</i>	-	+	-	61.0 ± 8.5	1.53 ± 0.12	1.52 ± 0.37
	<i>E4e5e10</i>	+	-	-	9.6 ± 4.6	41.01 ± 2.73	10.32 ± 3.16
	<i>e4e5e10</i>	-	-	-	63.1 ± 3.9	0.40 ± 0.03	0.44 ± 0.14

Competition of Four Alkaloids for Nitrosation Reaction



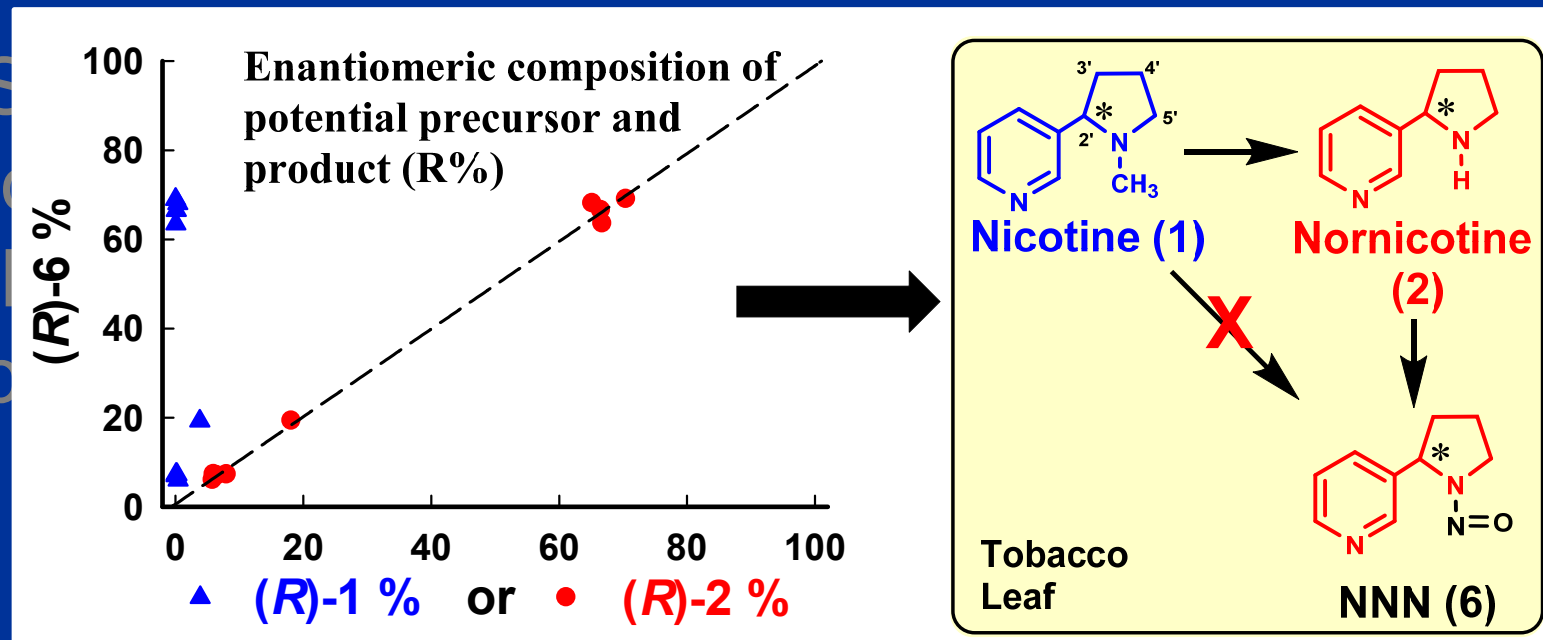
The presence of CYP82E4 will decrease the sensitivity of the NNK, NAT and NAB to their corresponding precursors

Summary

- ❖ A gas chromatography/thermal energy analyzer method using two columns in series was developed to separate the enantiomers of NNN, N'-nitrosoanabasine, and N'-nitrosoanatabine.
- ❖ In mutant lines, the concentration of NNN ranged from 0.44 $\mu\text{g g}^{-1}$ to 13.63 $\mu\text{g g}^{-1}$; (*R*)-NNN ranged from 0.08 $\mu\text{g g}^{-1}$ to 1.01 $\mu\text{g g}^{-1}$ and (*S*)-NNN from 0.34 $\mu\text{g g}^{-1}$ to 9.55 $\mu\text{g g}^{-1}$

Summary

- ❖ Based on the pattern of the enantiomeric composition, nicotine is not involved in direct formation of NNN in air-cured tobacco and cigarette smoke.



Correlations between NNN and its putative precursors

Correlation with NNN	Material
Nicotine	Burley¹⁵
+	
r = 0.66 **	Air-cured burley ⁴
r = 0.49	Commercial tobacco product ⁴¹
r = 0.40 NS	Flue-cured tobacco ²
r = 0.29 ** ^a	Air-cured dark tobacco leaf ⁷
r = 0.28 **	Air-cured Virginia tobacco ⁵
r = -0.65 *	Experimental cigarette ⁴²
Nornicotine	Burley¹⁵
+	
r = 0.95 **	Flue-cured tobacco ²
r = 0.52 **	Air-cured burley ⁴
r = 0.36 ** ^a	Air-cured dark tobacco leaf ⁷
r = 0.10 NS	Air-cured Virginia tobacco ⁵
Nitrate	
r = 0.99 **	Experimental cigarette ⁴²
r = 0.77 ** ^a	Commercial cigarette ⁴³
r = 0.77	Commercial tobacco product ⁴¹
r = 0.38 ** ^a	Air-cured, flue-cured, burley and dark tobacco ⁴⁴
r = 0.27 **	Air-cured flue tobacco ⁵
r = -0.01 NS ^a	Air-cured dark tobacco leaf ⁷
r = -0.12 NS	Air-cured burley ⁴
Nitrite	
r = 0.82 **	Air-cured flue, burley and dark tobacco ⁴⁴
r = 0.77 **	Air-cured burley ⁴⁵
r = 0.42 ** ^a	Air-cured dark tobacco leaf ⁷
r = -0.28 *	Air-cured burley ⁴

The results of this talk has been published (Cai, B.; Ji, H.; Fannin, F. F.; Bush, L. P. Contribution of nicotine and nornicotine toward the production of N'-nitrosonornicotine in air-cured tobacco (*Nicotiana tabacum*). J. Nat. Prod. 2016, 79 (4), 754–759.)

Summary

- ❖ Based on the pattern of the enantiomeric composition, nicotine is not involved in direct formation of NNN in air-cured tobacco and cigarette smoke.
- ❖ Since (*S*)-NNN is dominate form in smokeless tobacco and more carcinogenic than its *R* form, the reduction of (*S*)-nornicotine should be a priority for the reduction of NNN.

Thanks!