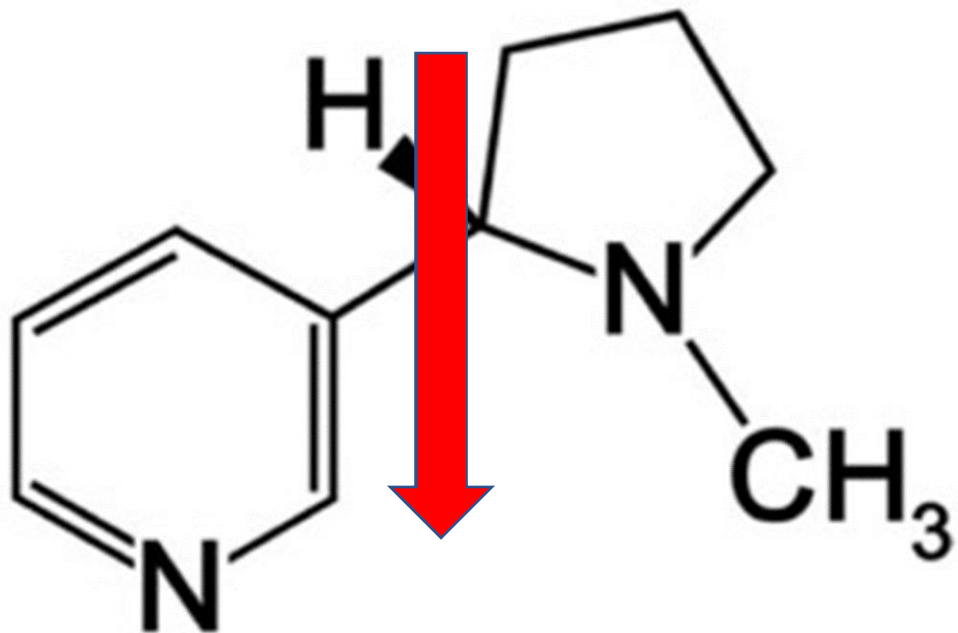




# POTENTIAL ULTRA-LOW NICOTINE LIMIT IN TOBACCO – CAN WE MEET IT?

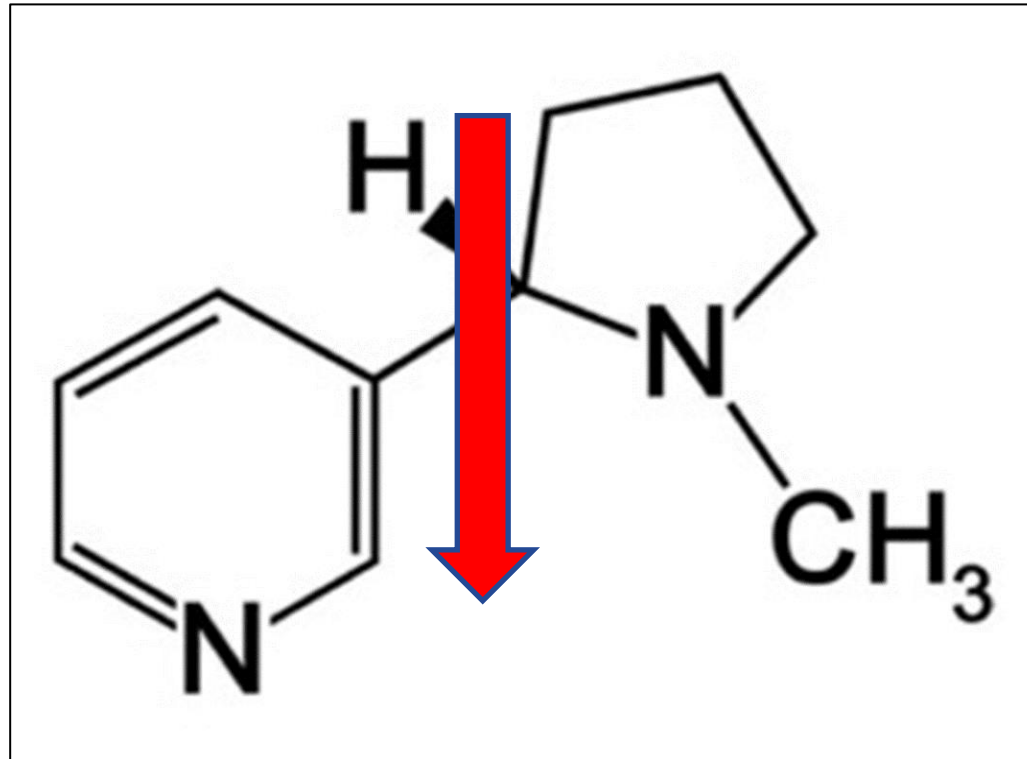


Anne Fisher, Colin Fisher, Barunava Patra, Huihua Ji,  
Jeffrey Kinney, Shengming Yang, Stacey Slone



# FDA ANPRM for Nicotine

- Lower nicotine to non-addictive levels
  - Possibly 0.3 – 0.5 mg/g in cigarette filler
  - Roughly 100x this level in commercial burley tobacco leaf







# Outline

- Nicotine limits
- Nicotine variability

## Strategies to reduce nicotine

- Modified agronomic practices
- Conventional LA mutants
- Combining conventional LA lines & agronomic practices
- Transgenics
- Gene-edited lines
- Conventional breeding – novel genes

# NICOTINE LIMITS





# Nicotine Limits

- ANPRM not a rule, not even a proposal
  - Requested comments on maximum limit 0.3 – 0.5 mg/g in filler
- Some controversy about non-addictive levels
- Technical feasibility?
  - Some evidence – biological limit to ↓ nicotine accumulation







# NICOTINE VARIABILITY



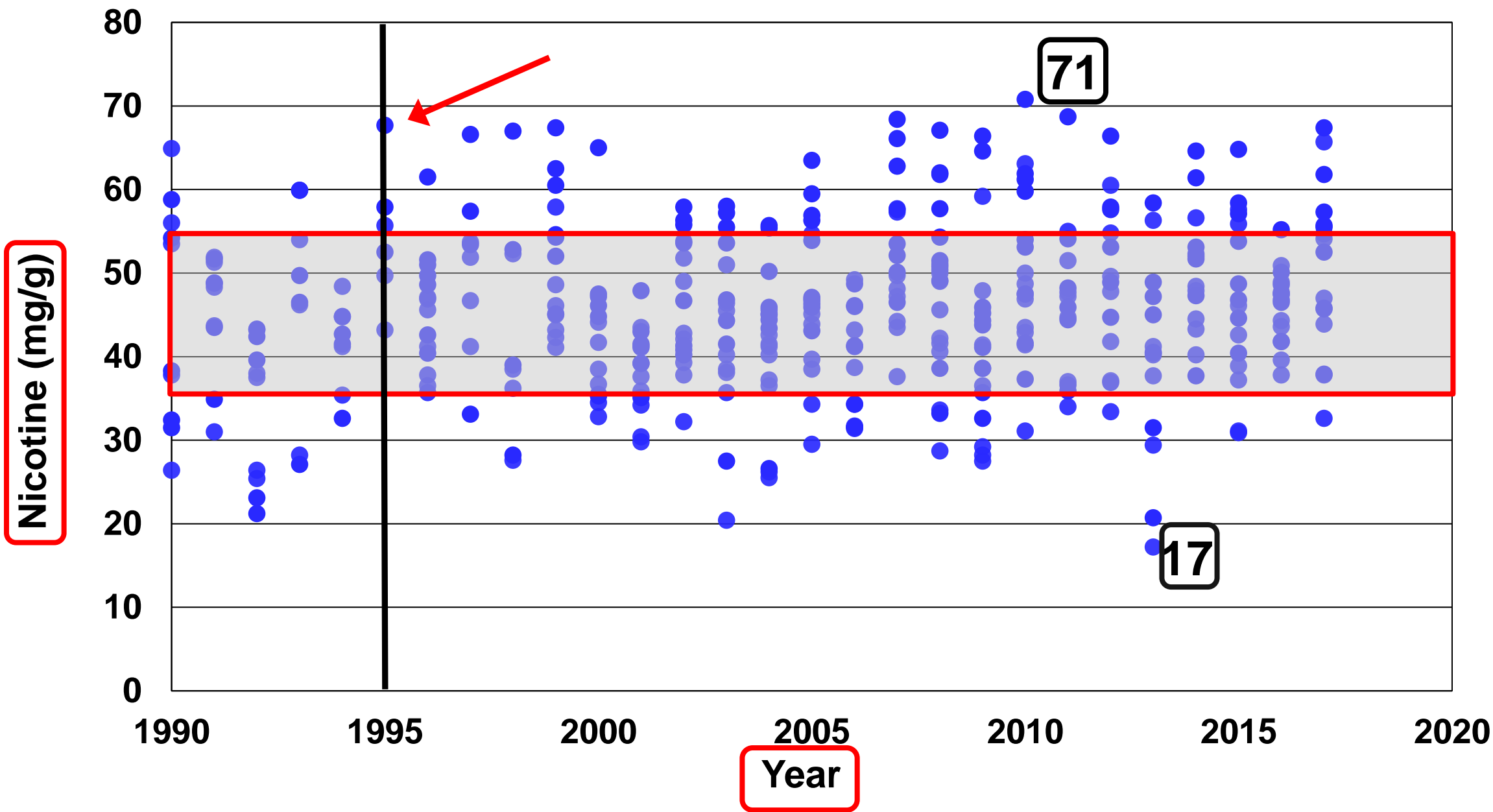
# Nicotine Variability

- Nicotine varies very widely, impacted by many factors
  - Season (weather)
    - Drier, ↑ nicotine
  - Location (soil, weather)
    - Heavier, more fertile soils, ↑ nicotine
  - Stalk position
  - Agronomic practices
- Minimum Standards Program (RQT) nicotine standard
  - Never absolute value, always % of checks
- Impossible to consistently meet an absolute value standard



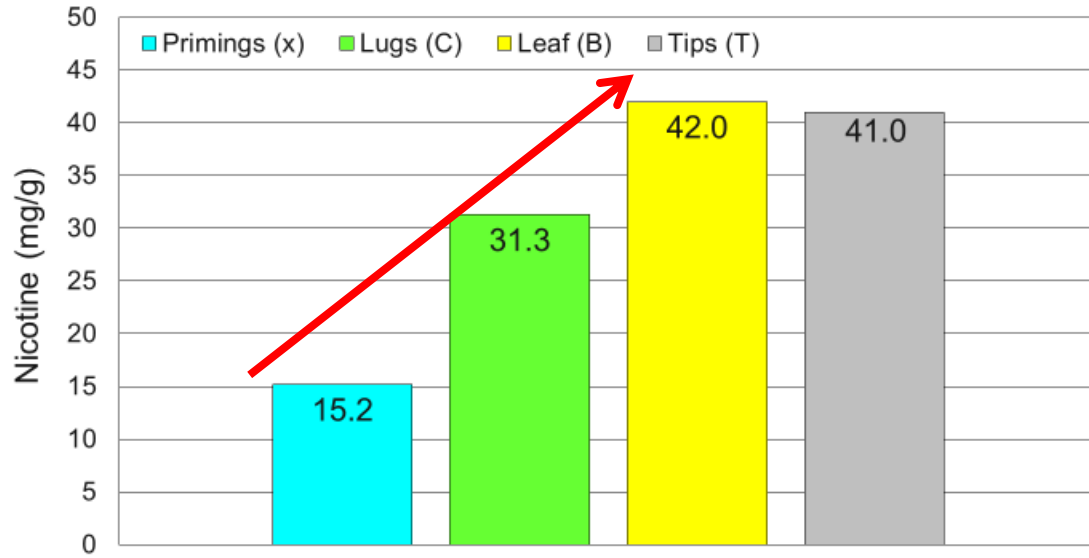


Season & Location: RQT Checks Nicotine (mg/g), 1990-2017

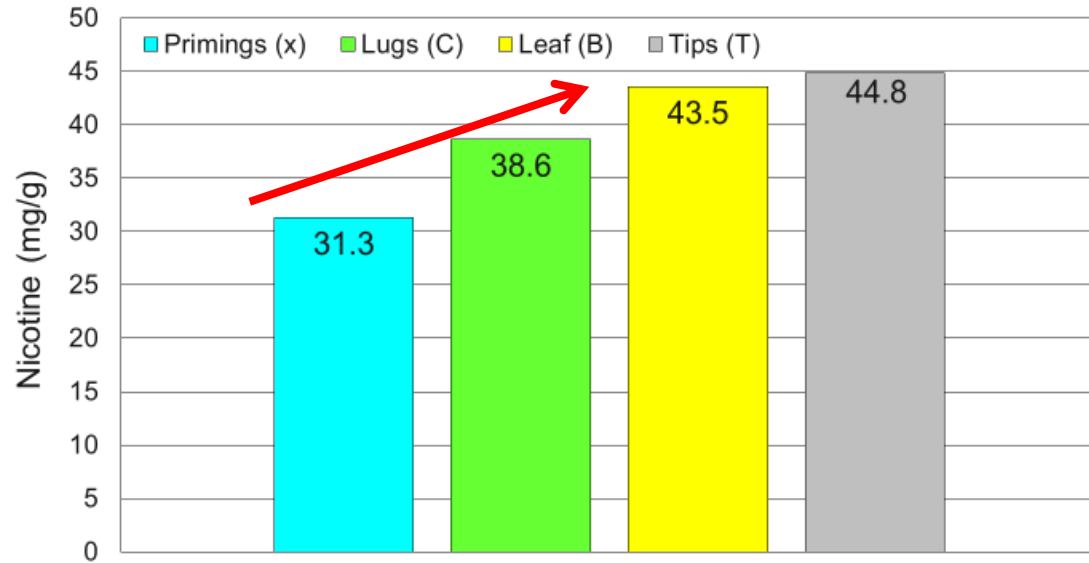




### Nicotine by Stalk Position (TN 90 2018)



### Nicotine by Stalk Position (Bu 21 2022)



## Stalk Position / Grade



**T** Primed Stalk-cut

**B** Stalk-cut

**C**

**X**







A photograph of two workers in a tobacco field. The worker on the left is wearing a light-colored shirt, blue jeans, a cap, and blue gloves, and is holding a large tobacco leaf. The worker on the right is wearing a light blue shirt, dark pants, and a cap, and is also working with the plants. The field is filled with rows of tobacco plants with large, green leaves. The background shows a line of trees under a clear sky.

# AGRONOMIC PRACTICES



# Agronomic Practices

Can lower nicotine – but often inconsistent

- Cannot lower nicotine anywhere near the target levels
- At best 50% for any one practice





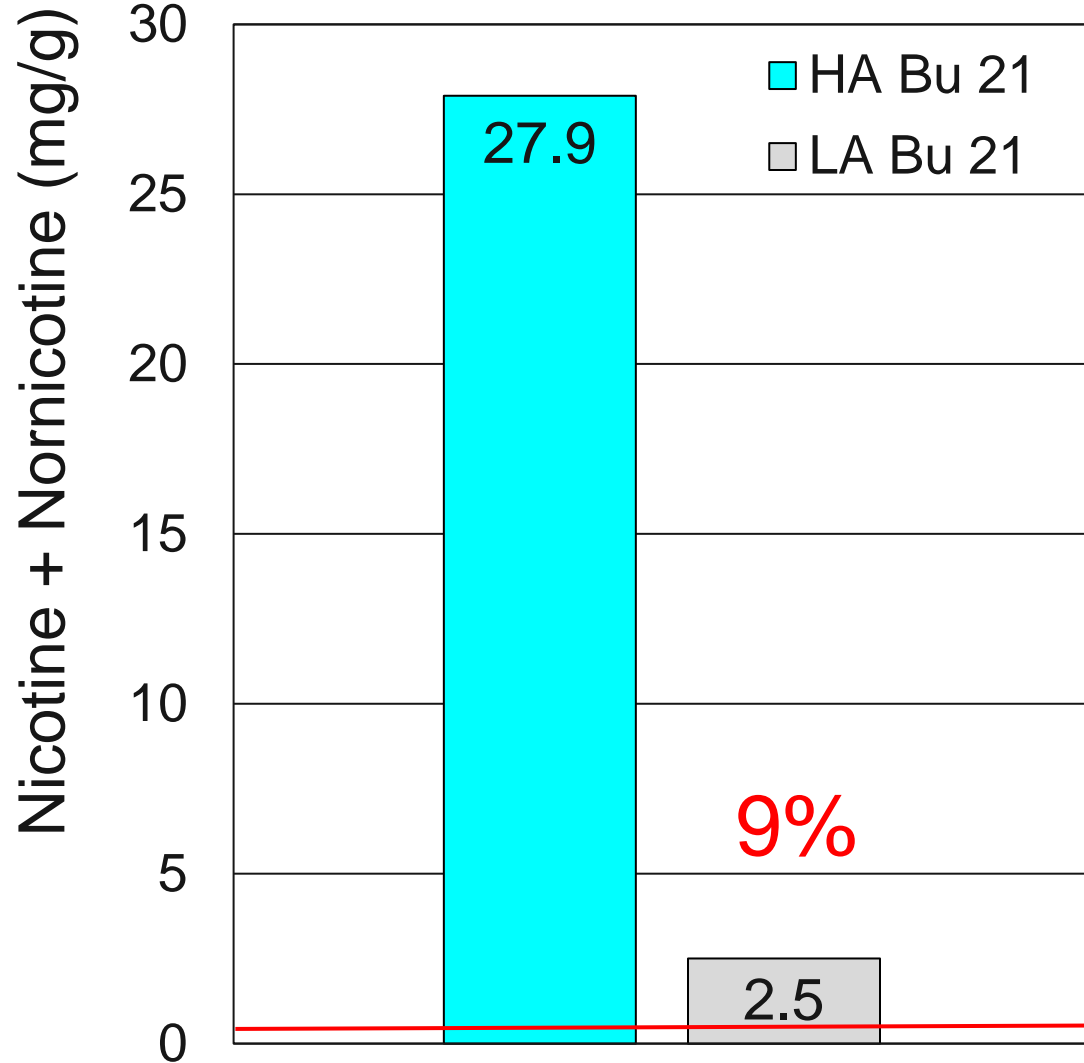


**CONVENTIONAL LA *nic1nic2*  
DOUBLE MUTANTS**

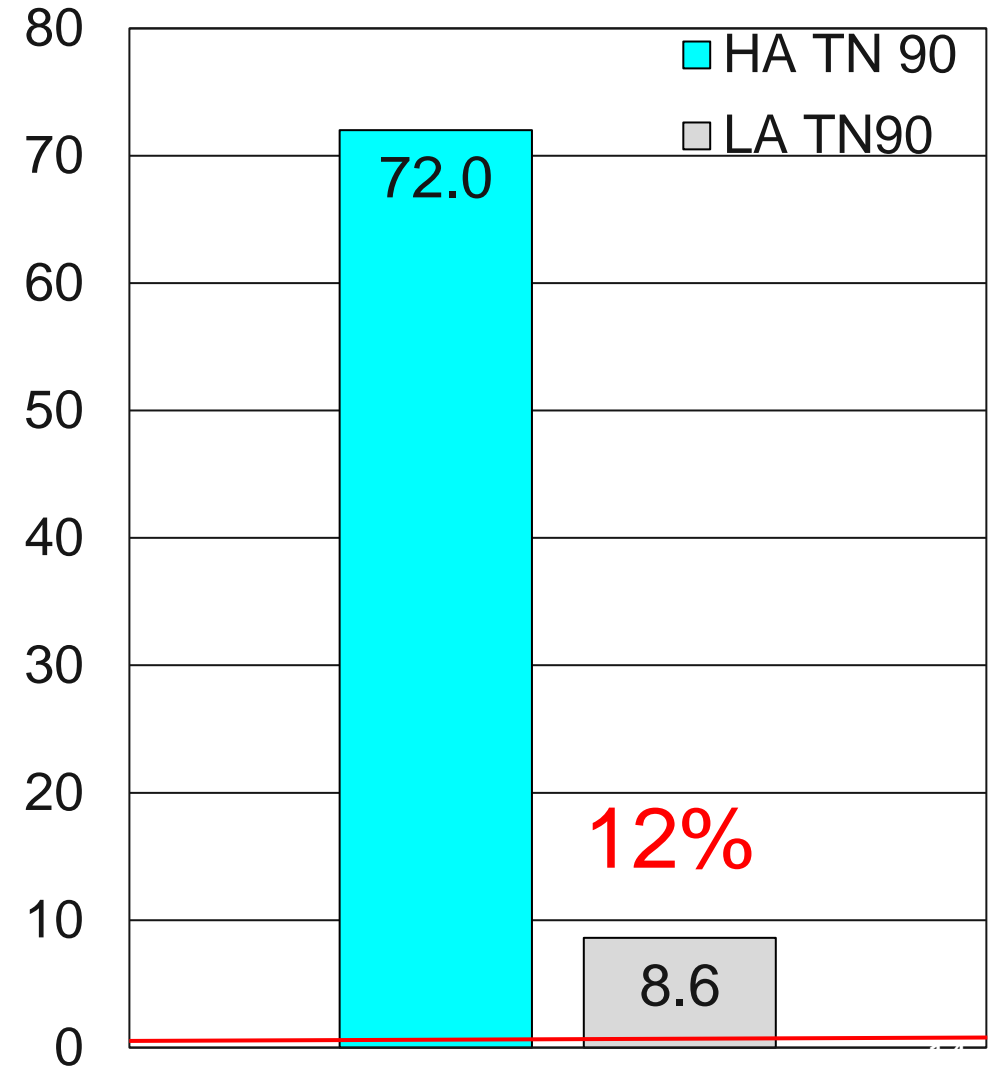


# Nicotine + Nornicotine (mg/g)

~10% Young Plants 2022



8-12% Cured Leaf, Overall 2019











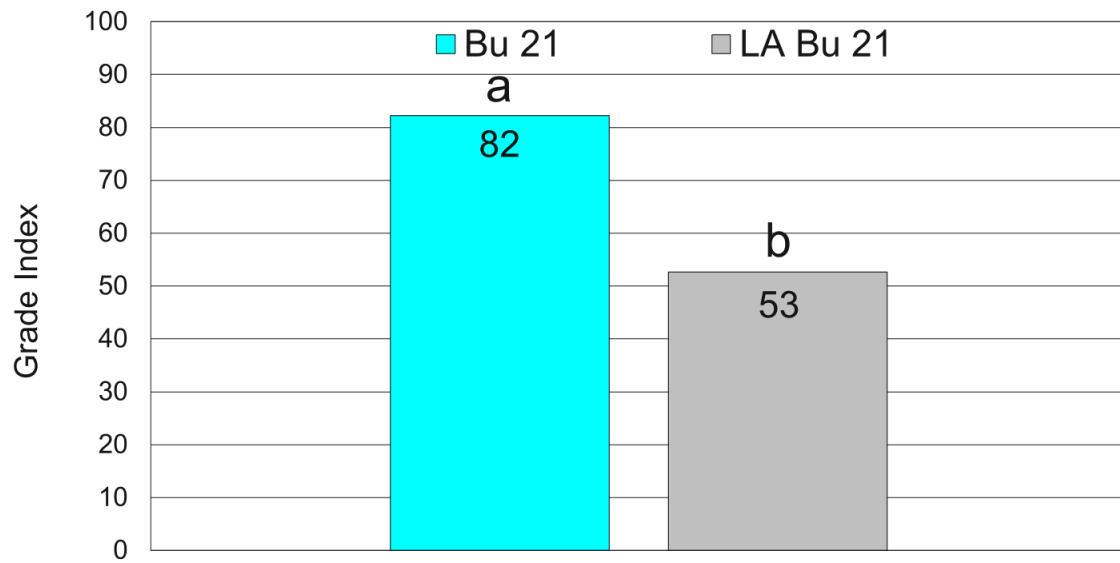
Bob Pearce

**LA**

**HA**



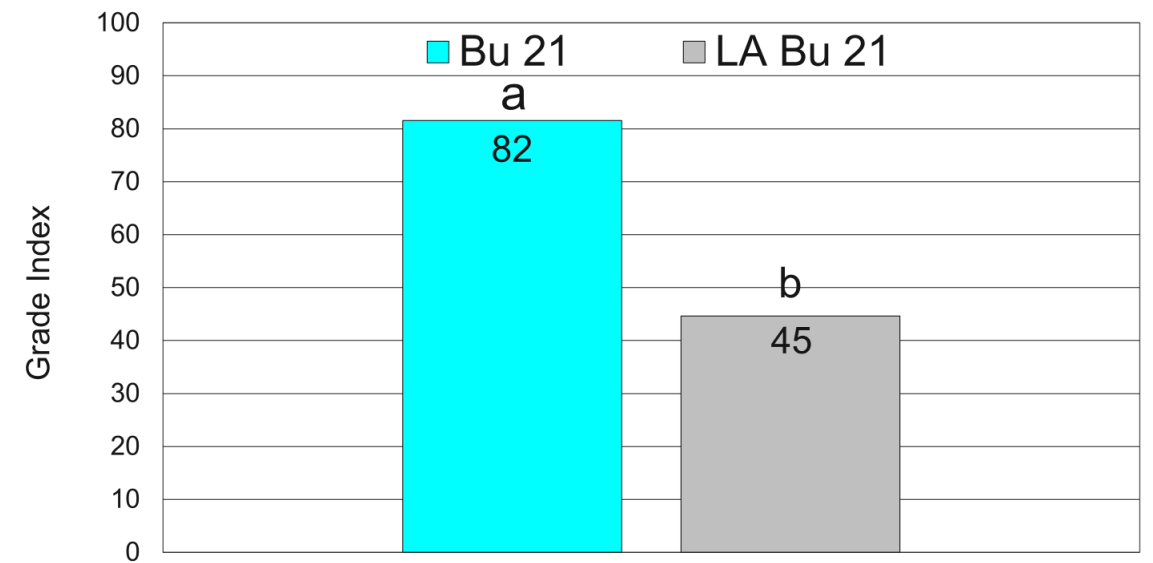
## Grade Index 2022



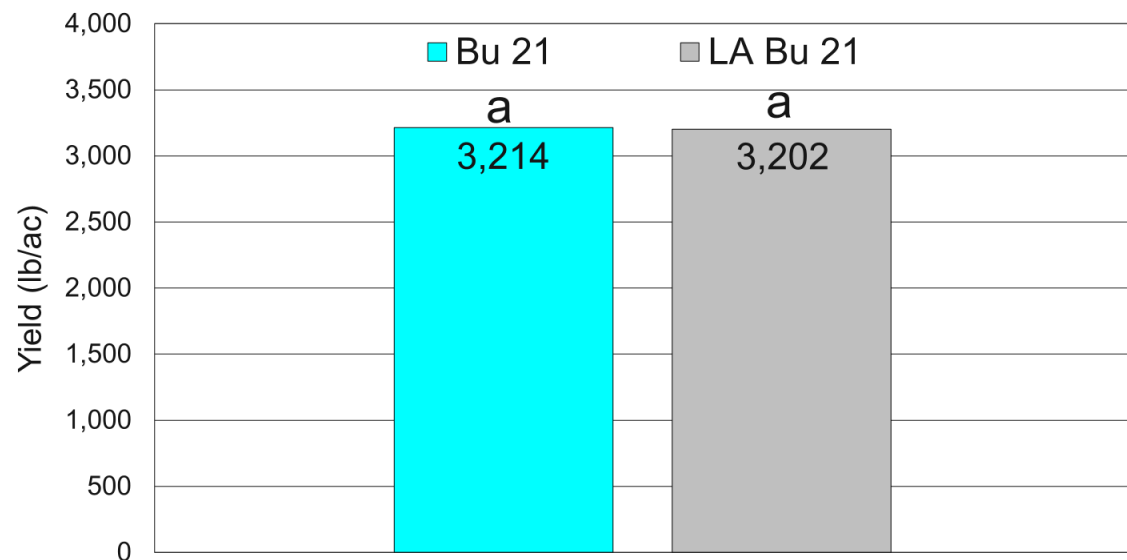
# LA Lines: Grade Index



## Grade Index 2023



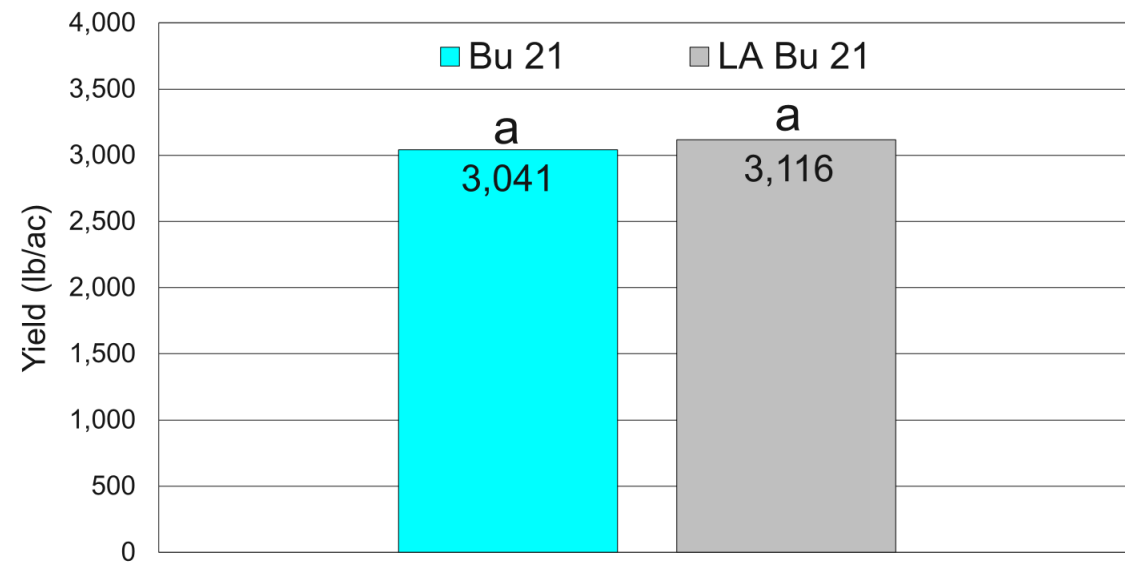
### Yield (lb/ac) 2022



# LA Lines: Yield



### Yield (lb/ac) 2023

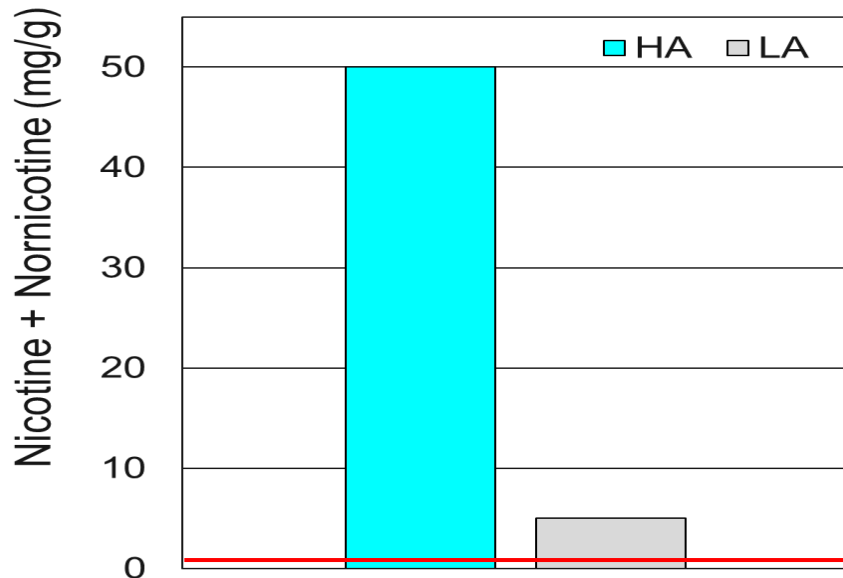




# Conventional Low Alkaloid Mutants

10% nicotine of std commercial varieties

- Still  $\uparrow$  10x higher than target
- Quality penalty  $\rightarrow$   $\downarrow$  return
- Insect problems – more sprays
  - $\uparrow$  costs,  $\downarrow$  return,  $\uparrow$  residues







# **COMBINING CONVENTIONAL LA MUTANTS & AGRONOMIC PRACTICES**



# Combined Effect of LA Lines & All Alkaloid Minimizing Agronomic Practices

- 2-year trial: 2018, 2019
  - HA check + 3 LA lines (*nic1nic2*)
  - Recommended agronomic practices & combination of all alkaloid minimizing practices

• Two VERY different seasons

• 2018 excellent early season

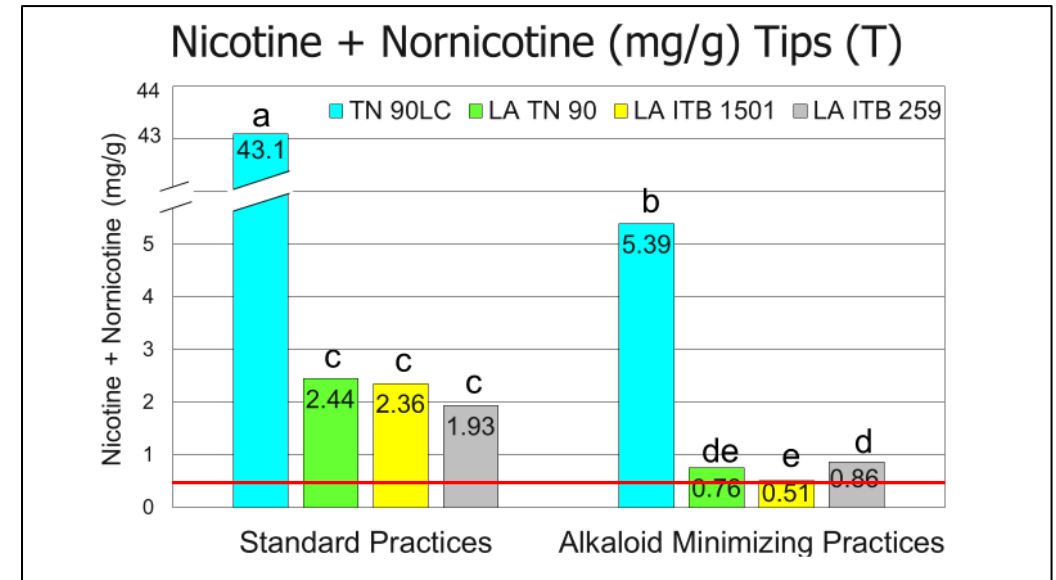
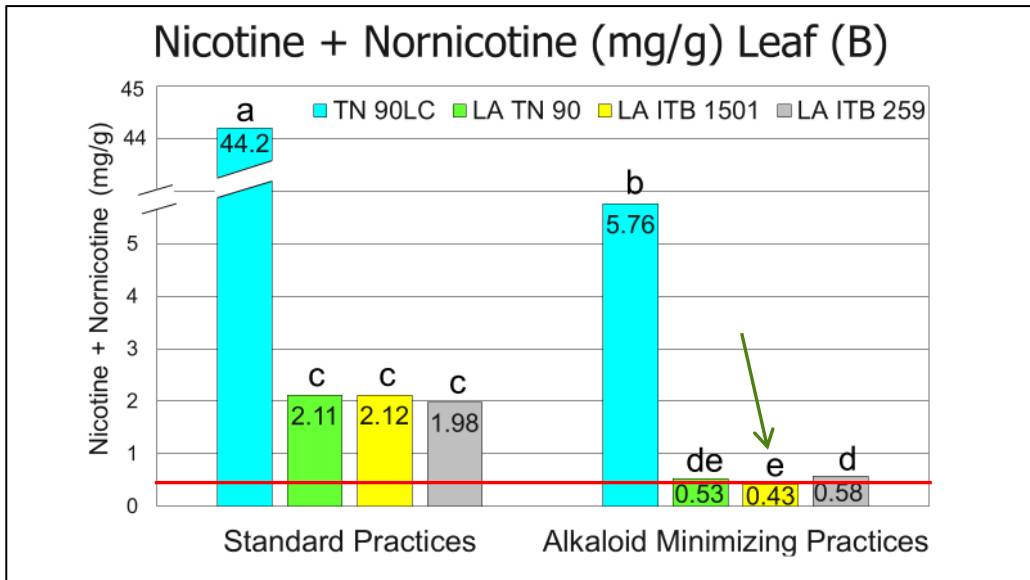
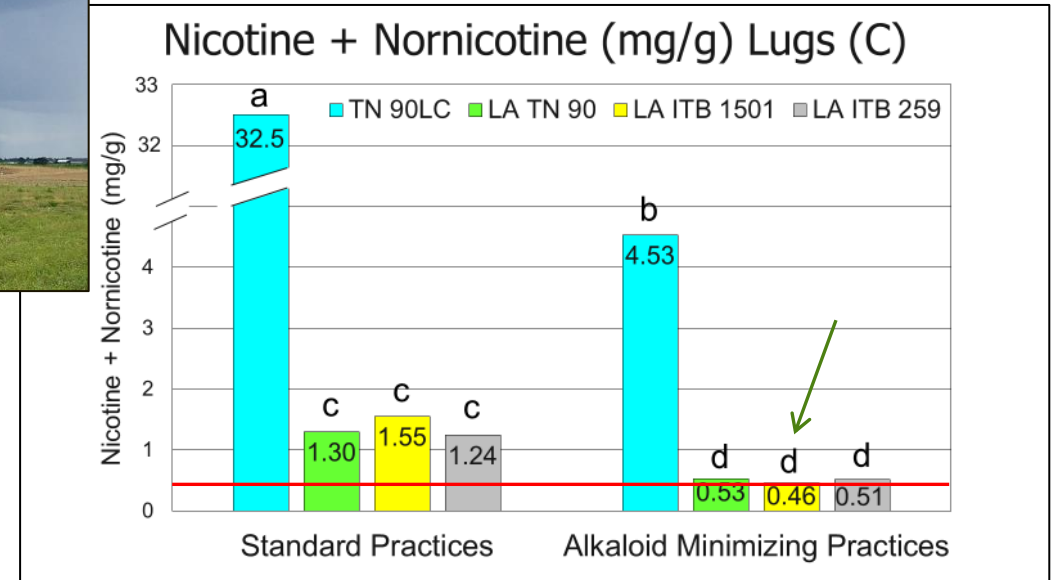
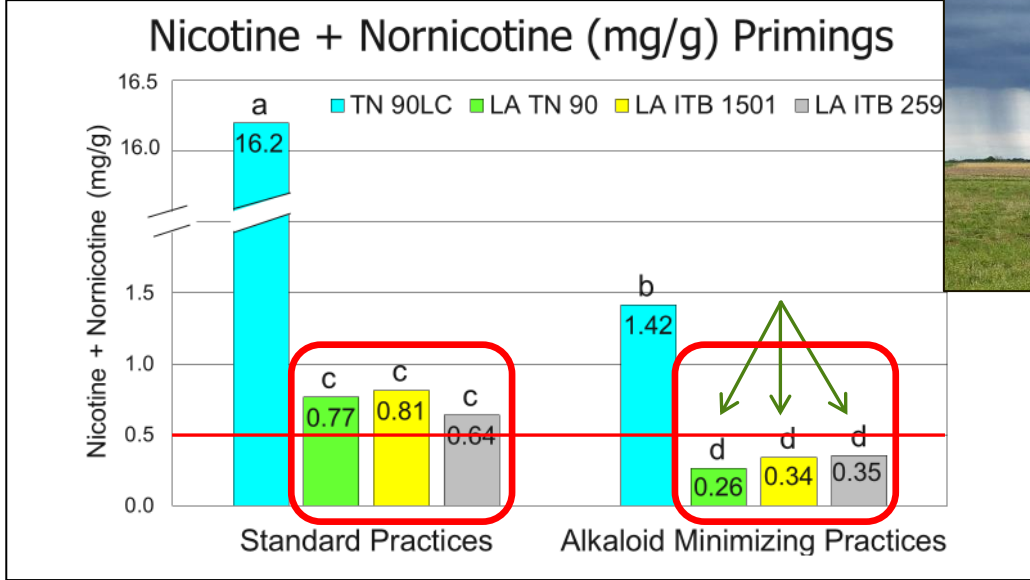
- Very low alkaloid (g/g)

• 2019 drought

- Very high alkaloid (g/g)

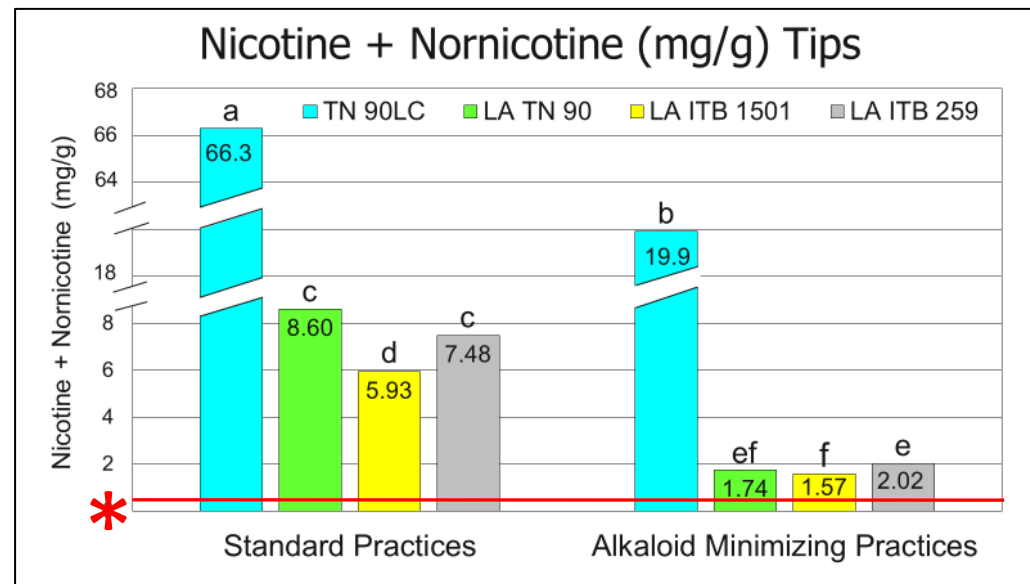
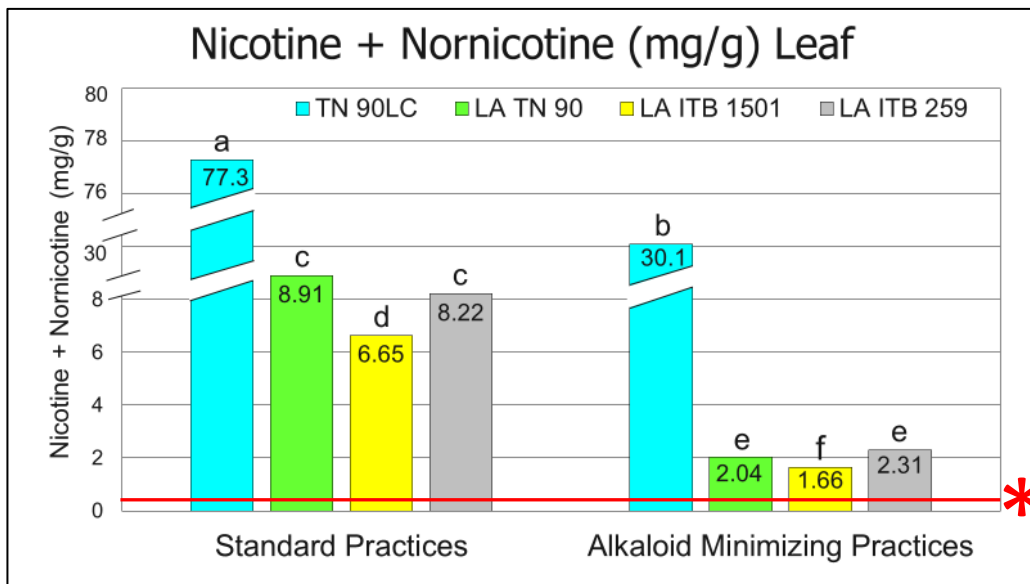
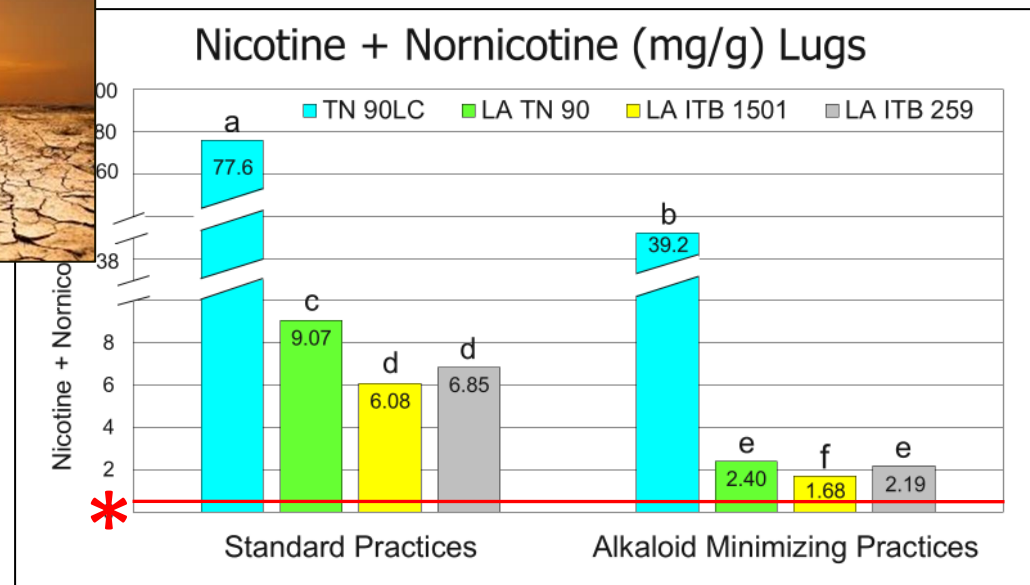
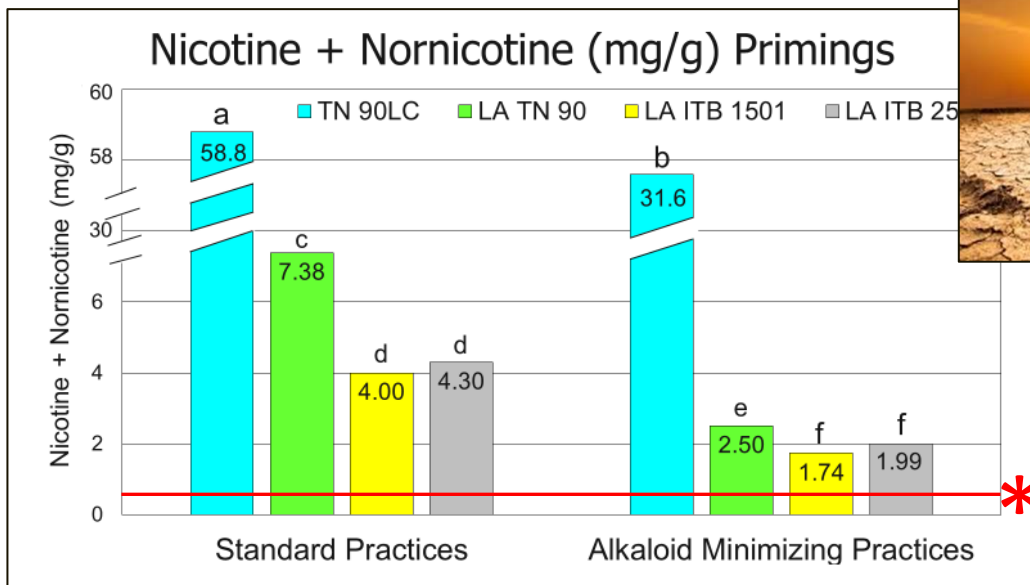


# 2018 Nicotine + Nornicotine (4 stalk Posns)





# 2019 Nicotine + Nornicotine (4 stalk Posns)



# LA Mutants + Agronomic Practices

*Some grades of some varieties just meet target in a VERY wet year: normal or dry season – all way above target*

- Cannot consistently meet target







# Transgenics

# Transgenics

- Only ultra-low nicotine transgenic in use – achieved lower nicotine using high converters
  - (VLN cigarettes >30% conversion in filler)
- Argument – still very low absolute amount of nornicotine
  - >0.2 mg/g nornicotine, >0.6 mg/g nicotine in filler (>0.5 mg/g)
  - Abuse liability of nornicotine – several papers show:
    - Nornicotine mimics nicotine in the body
    - Substitutes for nicotine in drug discrimination testing
  - Defeats the objective of non-addictive levels



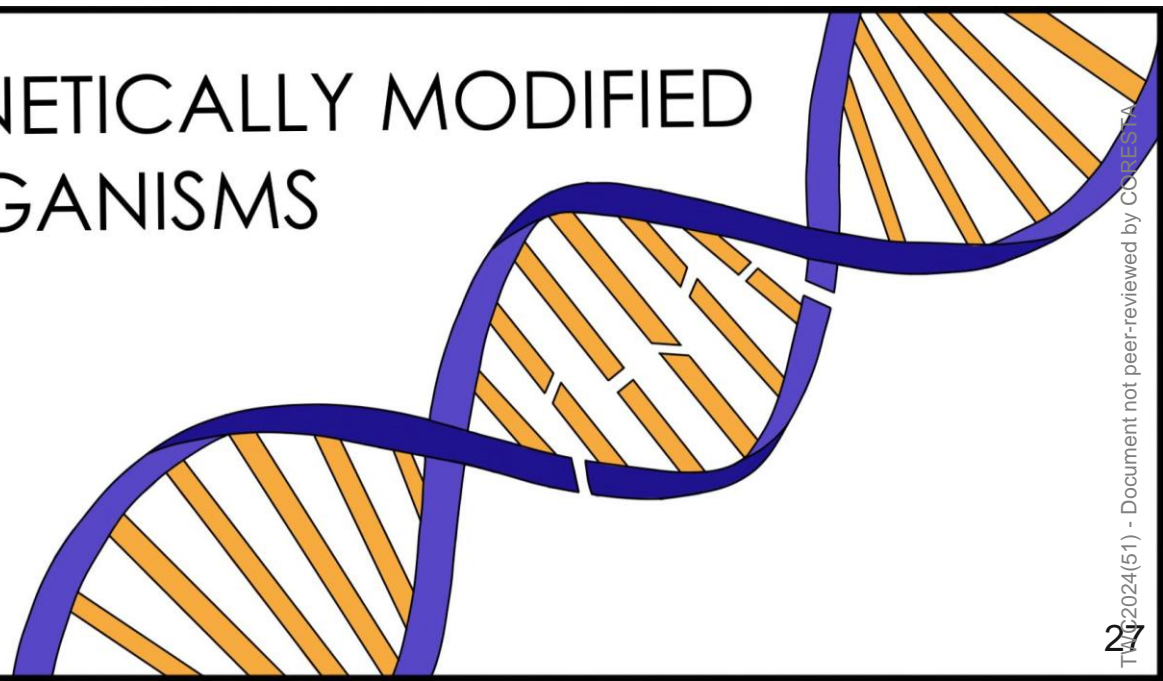
# Transgenics

Transgenics are a means to an end, not the end

- Not accepted by tobacco industry
- Only ultra-low transgenic tobacco in use has high conversion – nornicotine abuse liability similar to nicotine
- Very low sales



GENETICALLY MODIFIED ORGANISMS





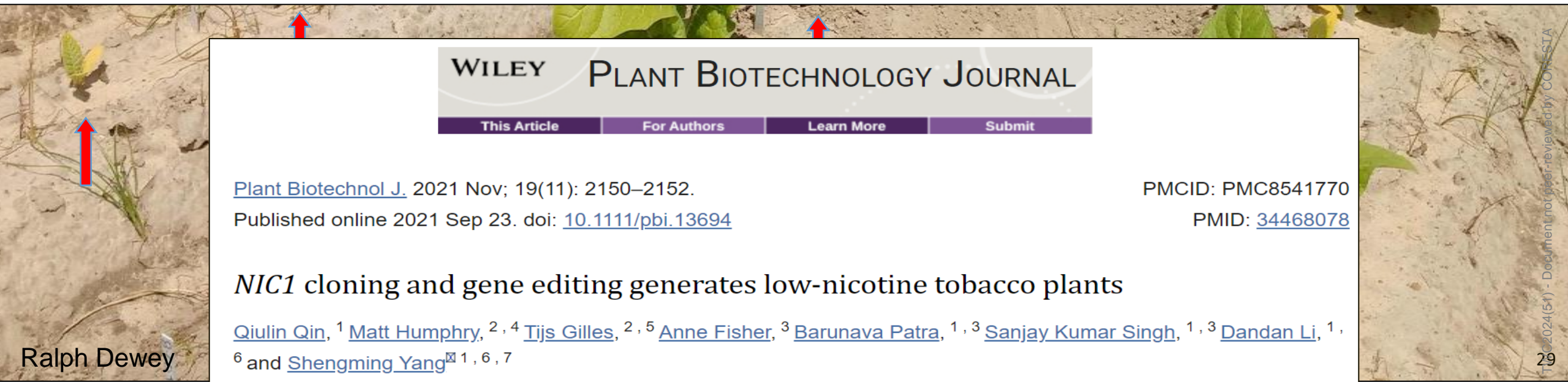


# GENE-EDITED LINES



# Gene-edited Lines

- Several ultra-low gene-edited lines developed
  - Some groups report severe growth deficiencies in the field
  - Qin *et al* edited *Nic1* in HI Bu 21 → lines with ultra-low nicotine and normal growth



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[Plant Biotechnol J.](#) 2021 Nov; 19(11): 2150–2152.

PMCID: PMC8541770

Published online 2021 Sep 23. doi: [10.1111/pbi.13694](https://doi.org/10.1111/pbi.13694)

PMID: [34468078](https://pubmed.ncbi.nlm.nih.gov/34468078/)

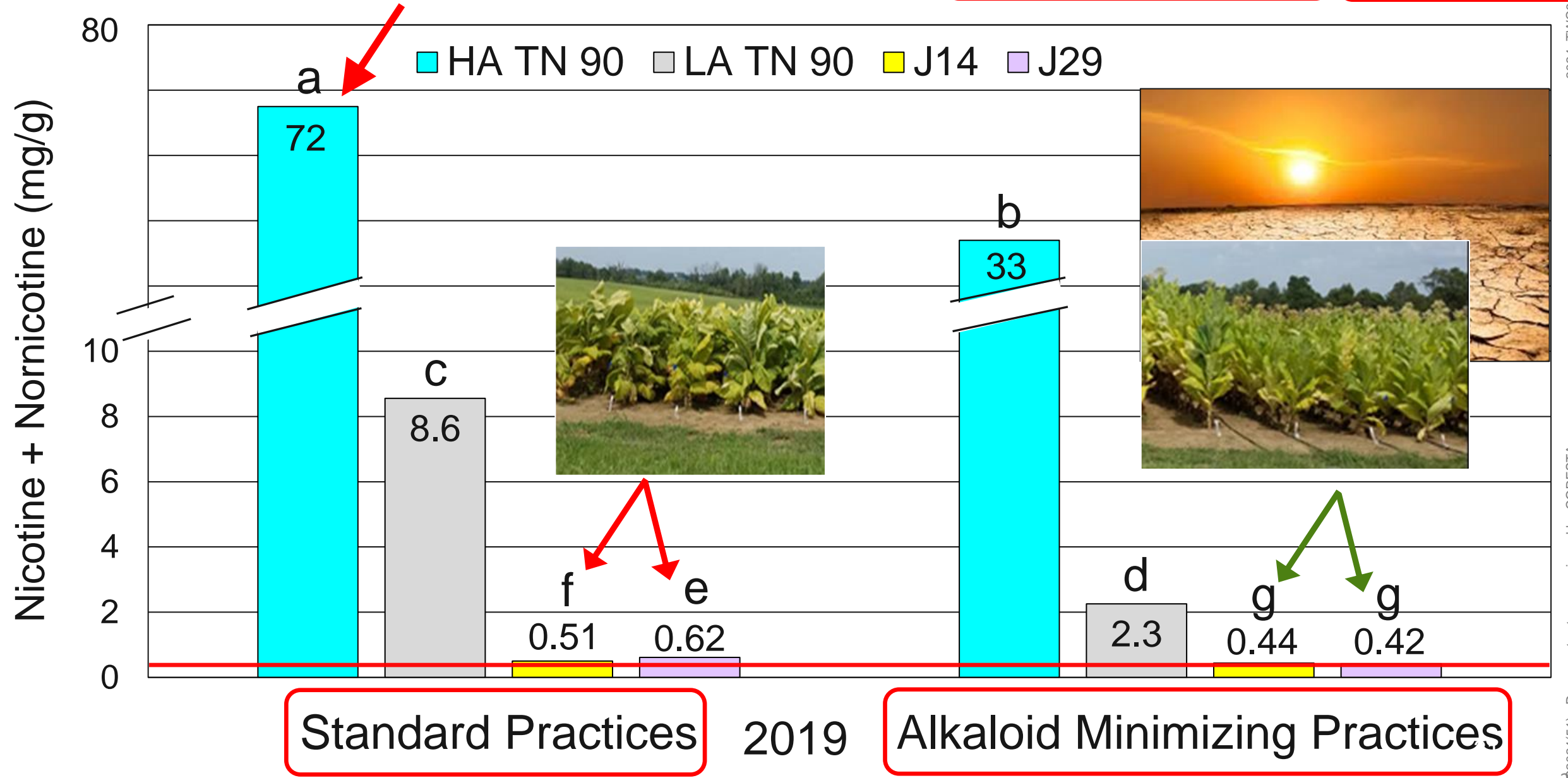
*NIC1* cloning and gene editing generates low-nicotine tobacco plants

[Qiulin Qin](#), <sup>1</sup> [Matt Humphry](#), <sup>2, 4</sup> [Tijs Gilles](#), <sup>2, 5</sup> [Anne Fisher](#), <sup>3</sup> [Barunava Patra](#), <sup>1, 3</sup> [Sanjay Kumar Singh](#), <sup>1, 3</sup> [Dandan Li](#), <sup>1, 6</sup> and [Shengming Yang](#) <sup>1, 6, 7</sup>

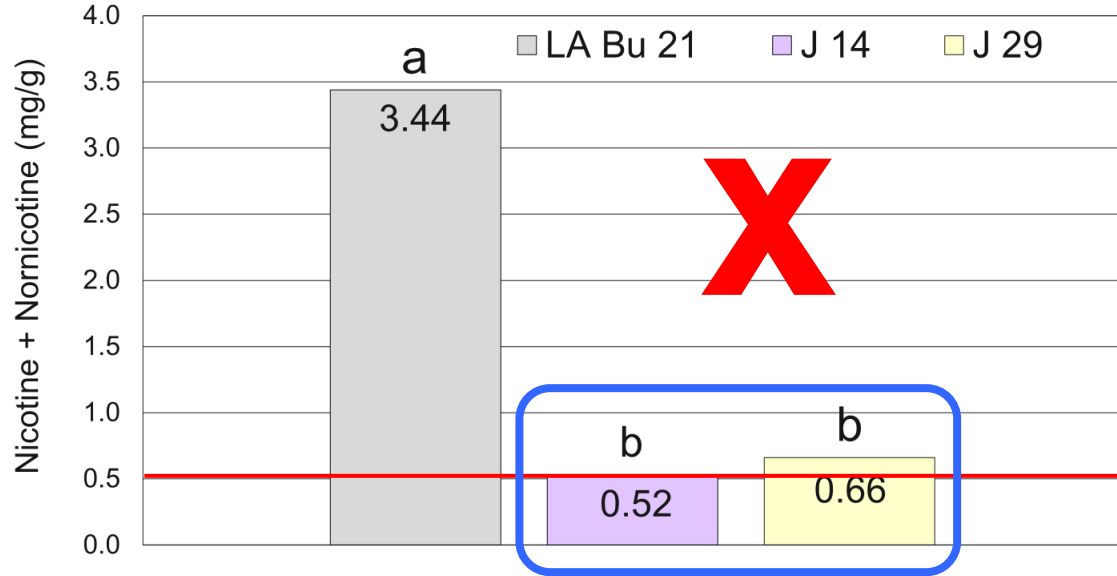




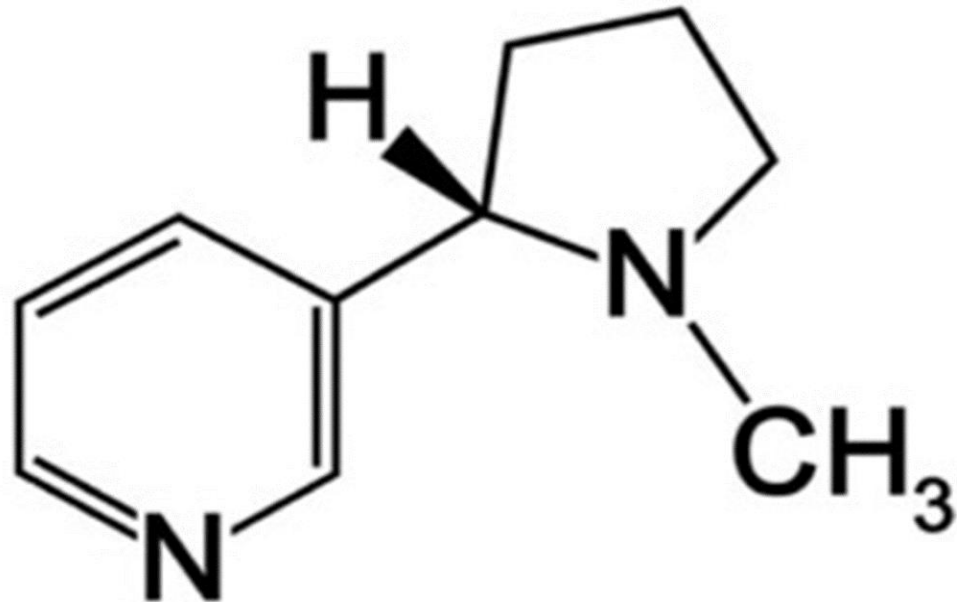
# Nicotine + Nornicotine (mg/g) Cured Leaf, Overall



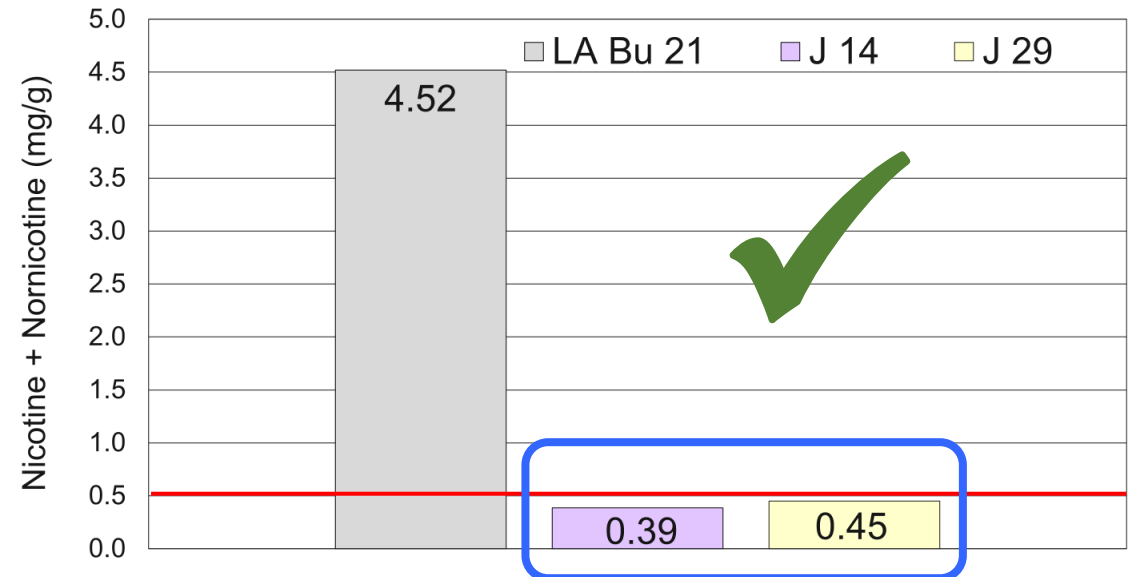
## Nicotine + Nornicotine (mg/g) Leaf (B) 2022



# Gene-Edited Lines: Nicotine + Nornicotine Leaf Grade

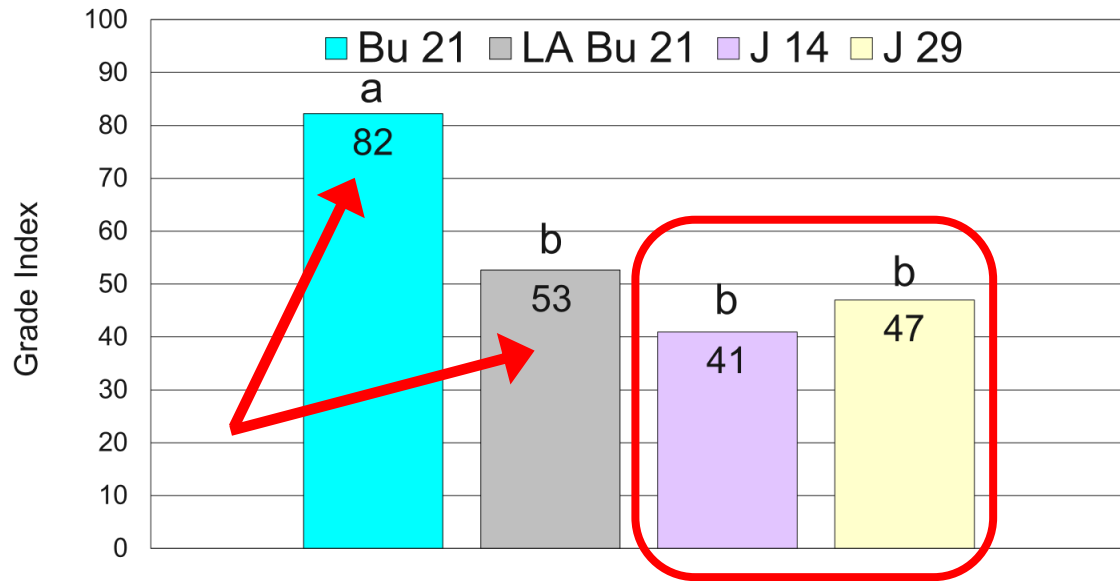


## Nicotine + Nornicotine (mg/g) Leaf (B) 2023





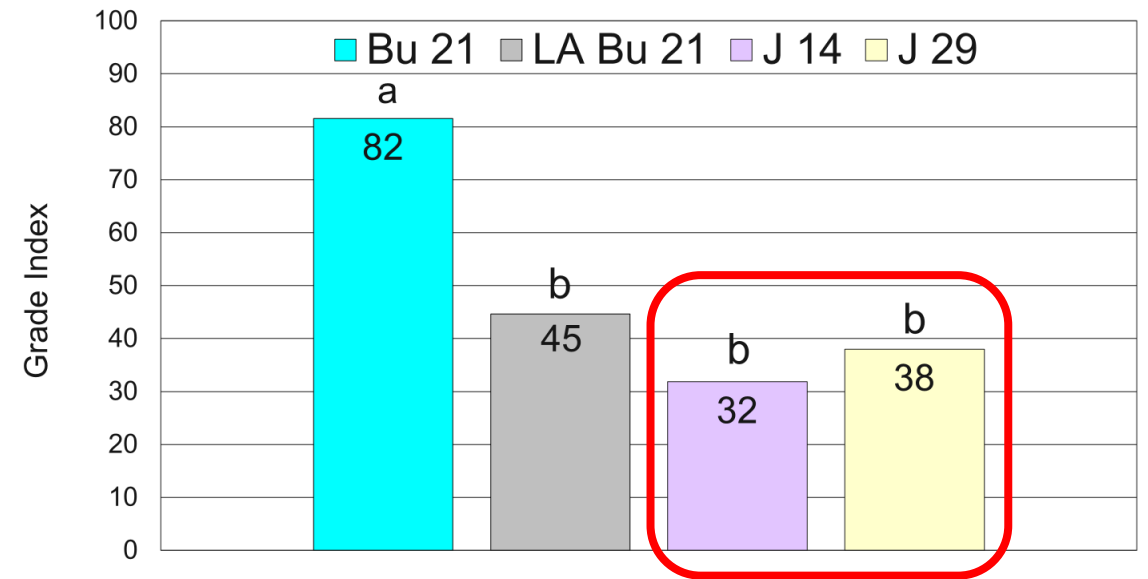
### Grade Index 2022



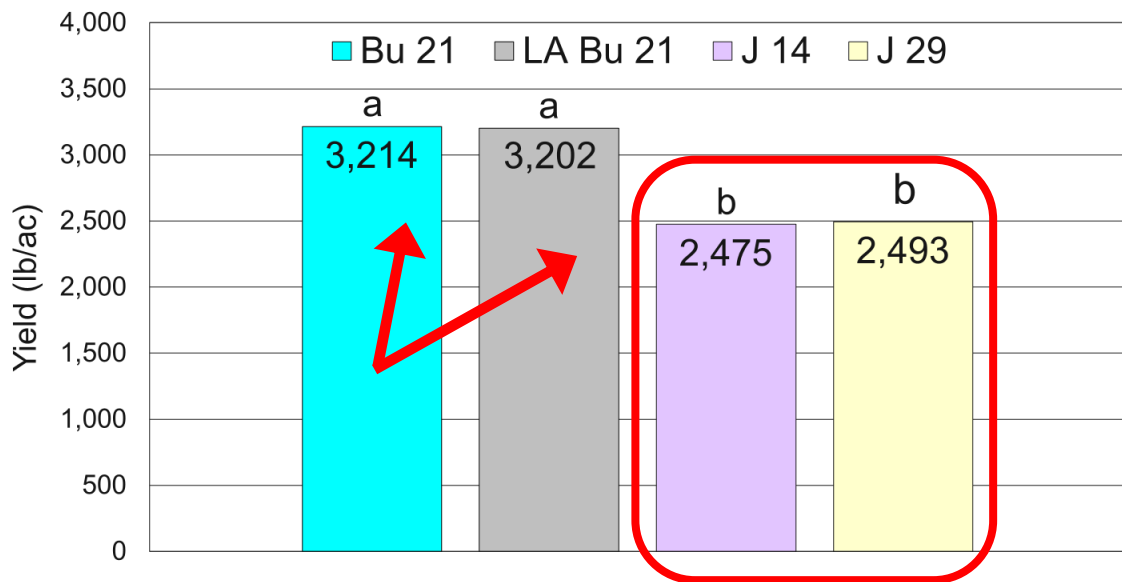
# Gene-Edited Lines: Grade Index



### Grade Index 2023



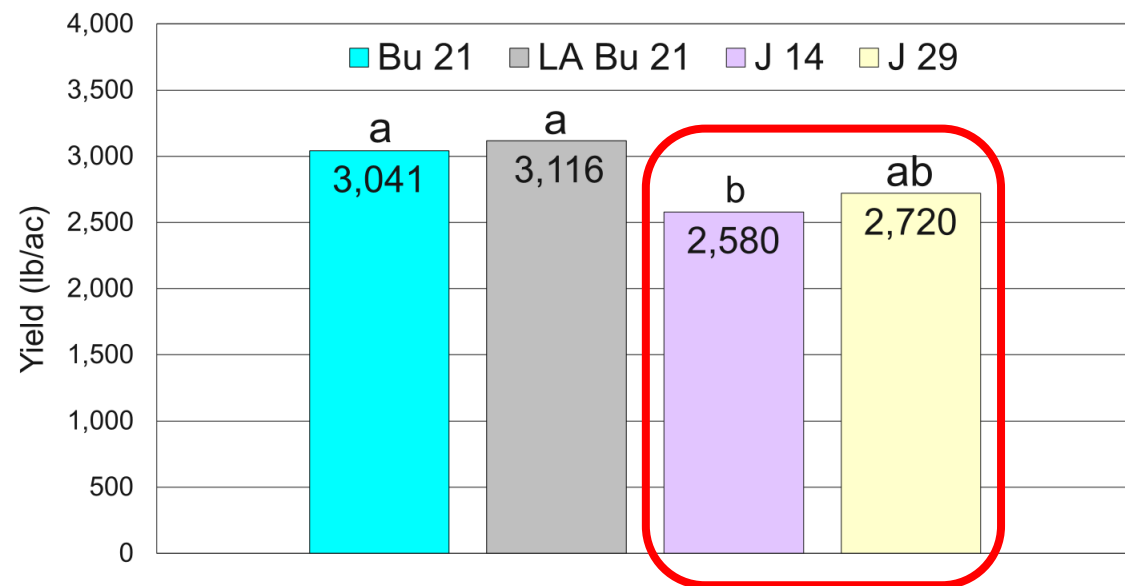
Yield (lb/ac) 2022



# Gene-Edited Lines: Yield



Yield (lb/ac) 2023





# Gene-Edited Lines

Even these lines cannot consistently meet target

- Some seasons, some grades – do meet target – but not all
- With alkaloid ↓ practices, did meet target in dry year (1 yr data)
- Gene editing in general, CRISPR in particular, currently not options for tobacco industry





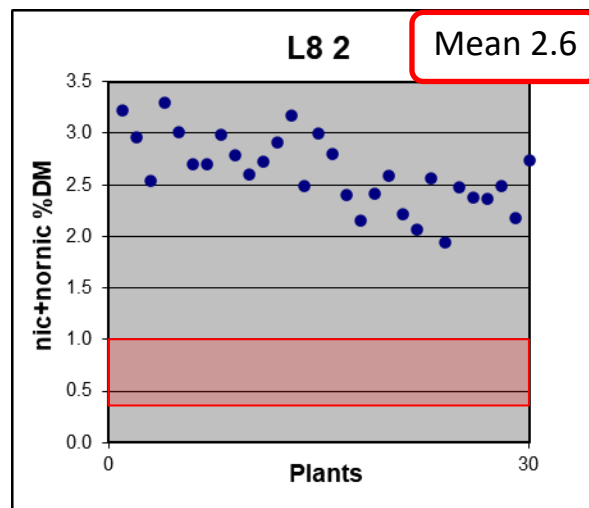
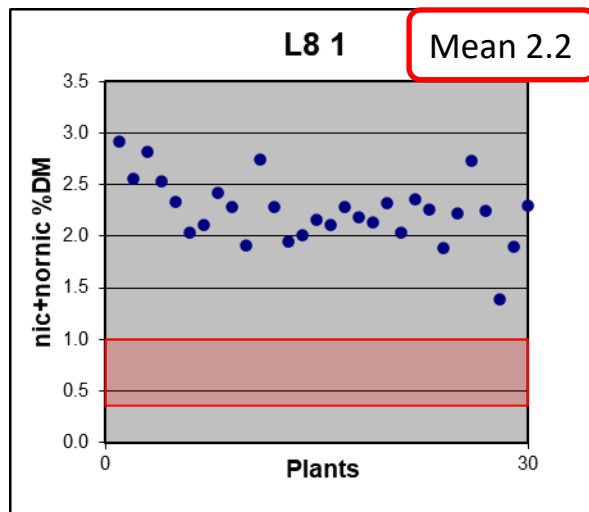


# NOVEL LOW NICOTINE GENE

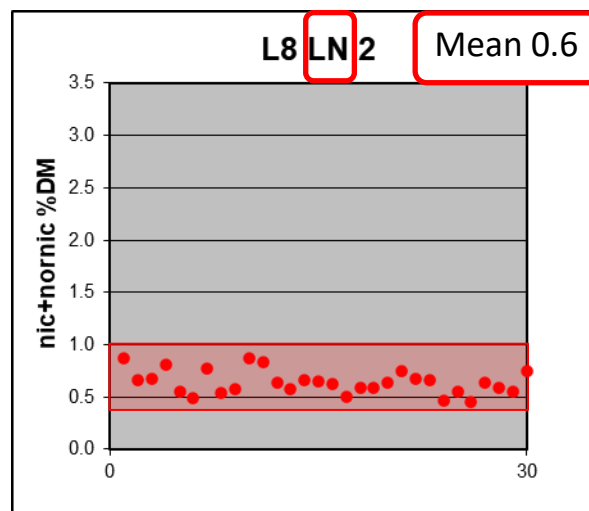
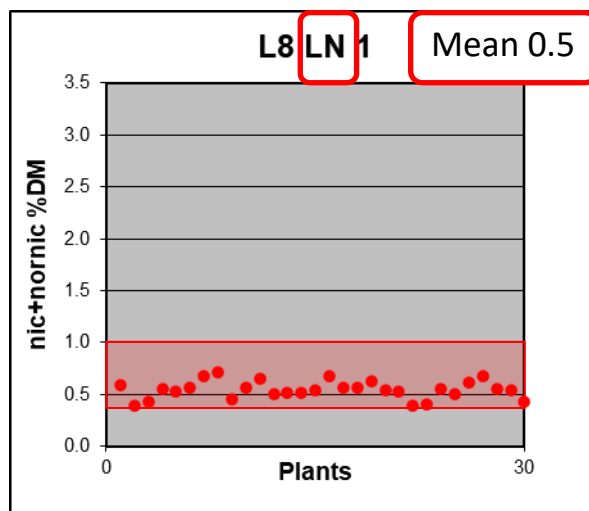


# Novel Low Alkaloid Gene in **L8**

**Spontaneous Mutation** – 2 Families Selected in Parallel from Same Base Population



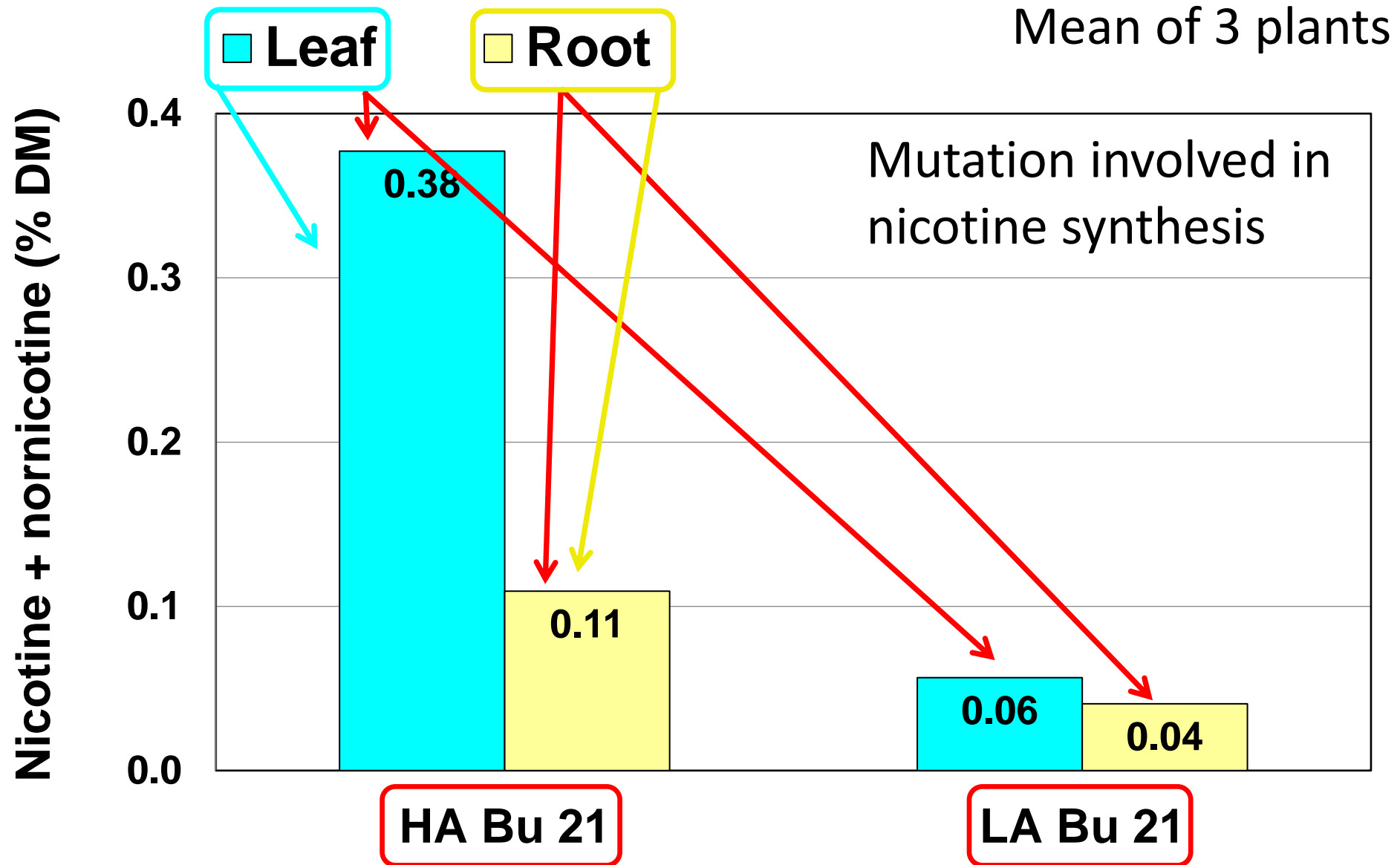
Normal nicotine family



- No overlap
- *Nic1Nic2* markers show same genotype in both families
  - *AAbb*, *H1*, *Nic1nic2*
  - Assume novel gene, designate LN

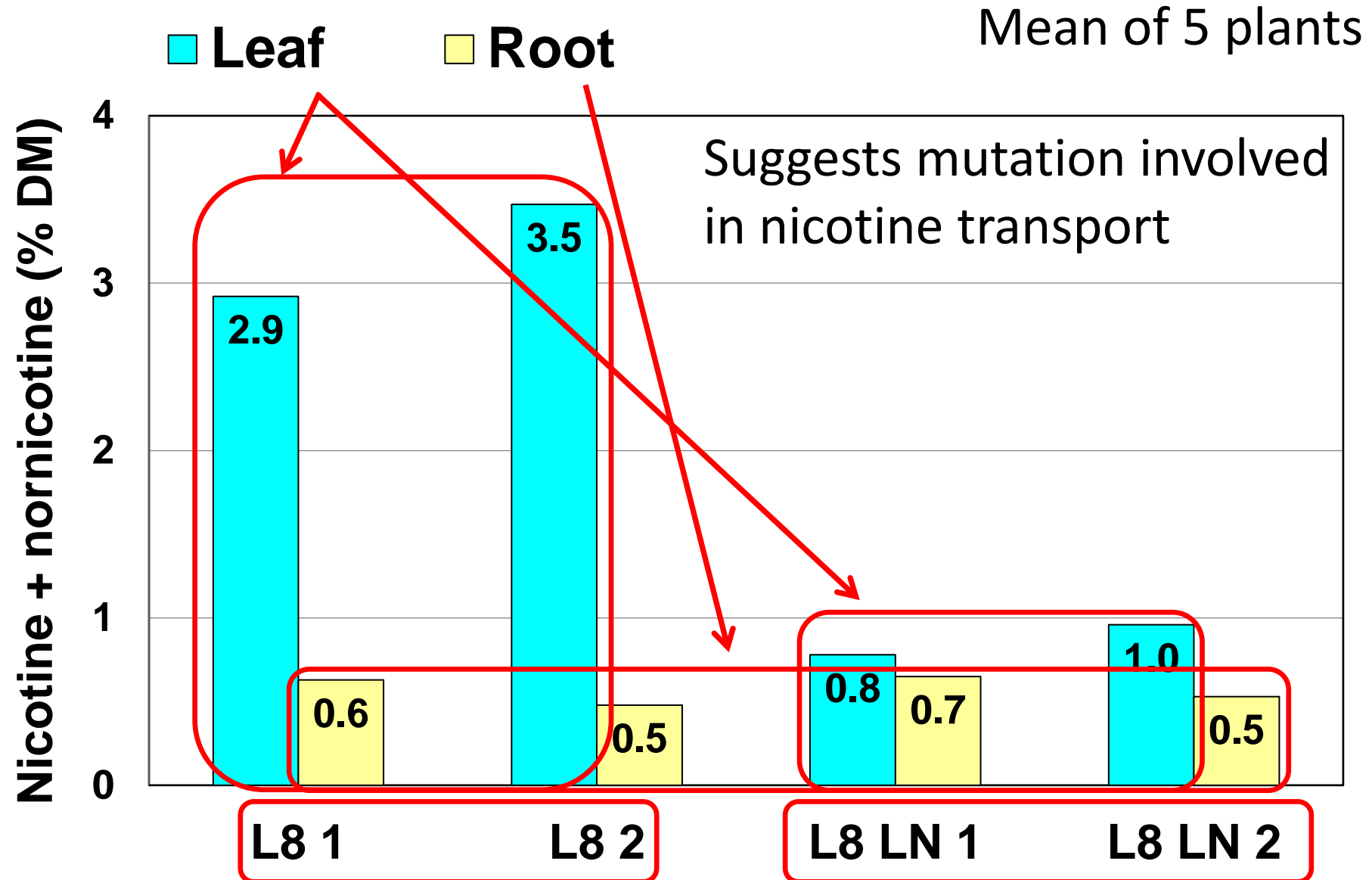
Lower nicotine family

# Nicotine + Nornicotine in Leaf and Root – HA vs LA

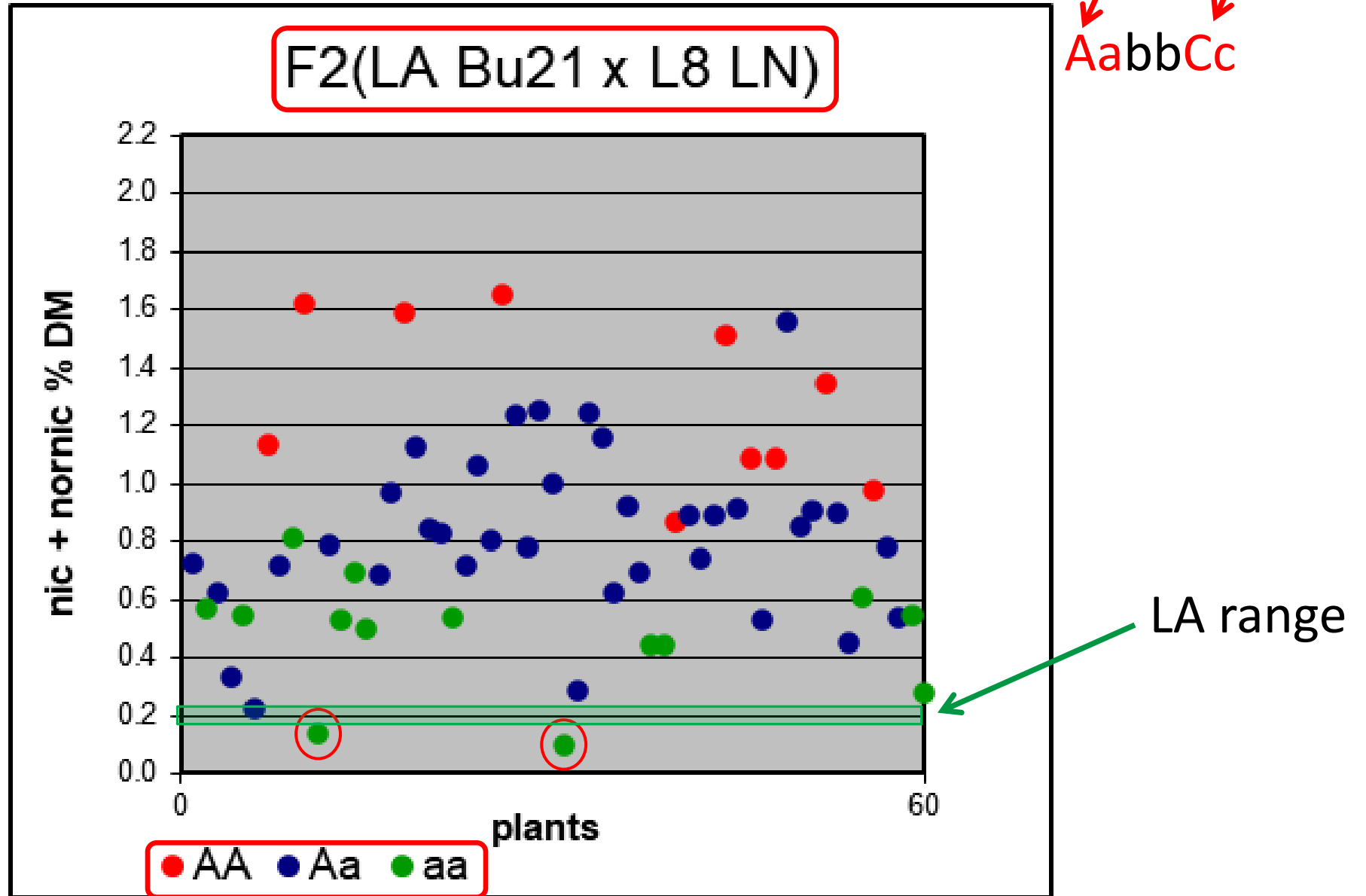




# Nicotine + Nornicotine in Leaf and Root – L8 Lines

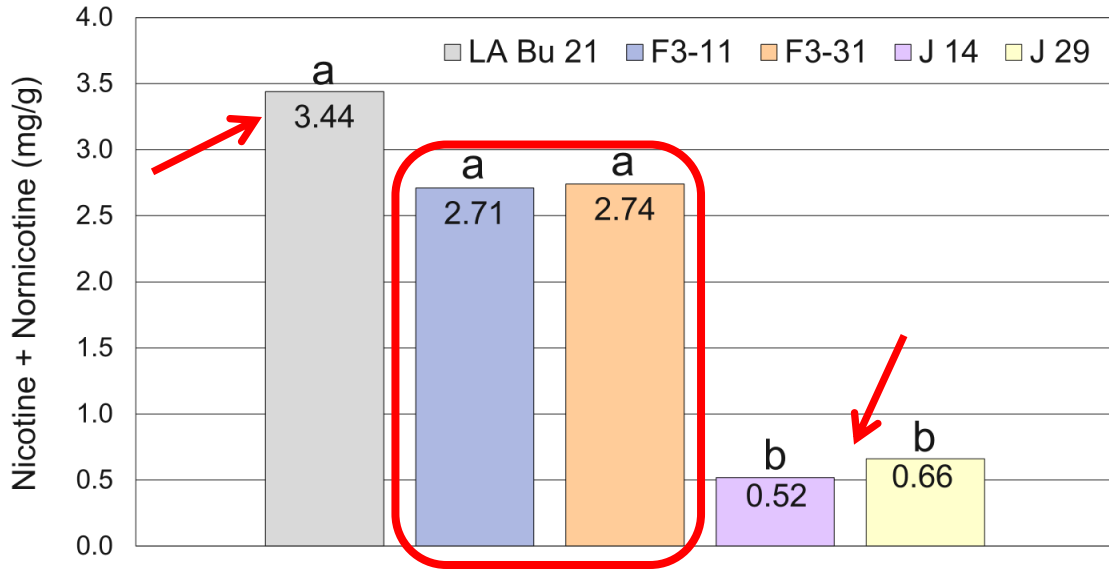


# Stacking LN with *nic1nic2*



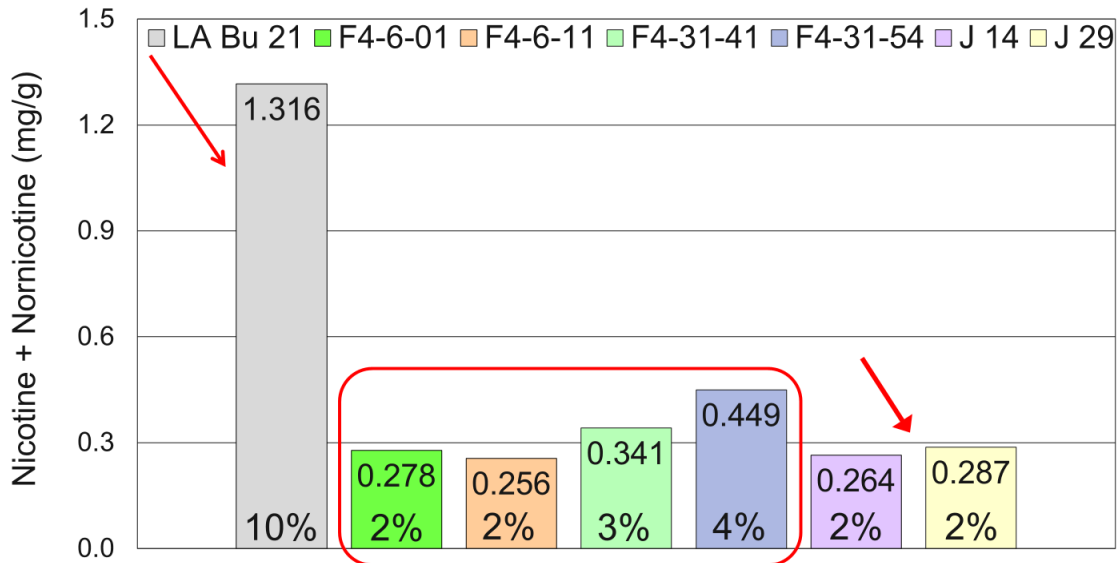


## Nicotine + Nornicotine (mg/g) Leaf (B) 2022

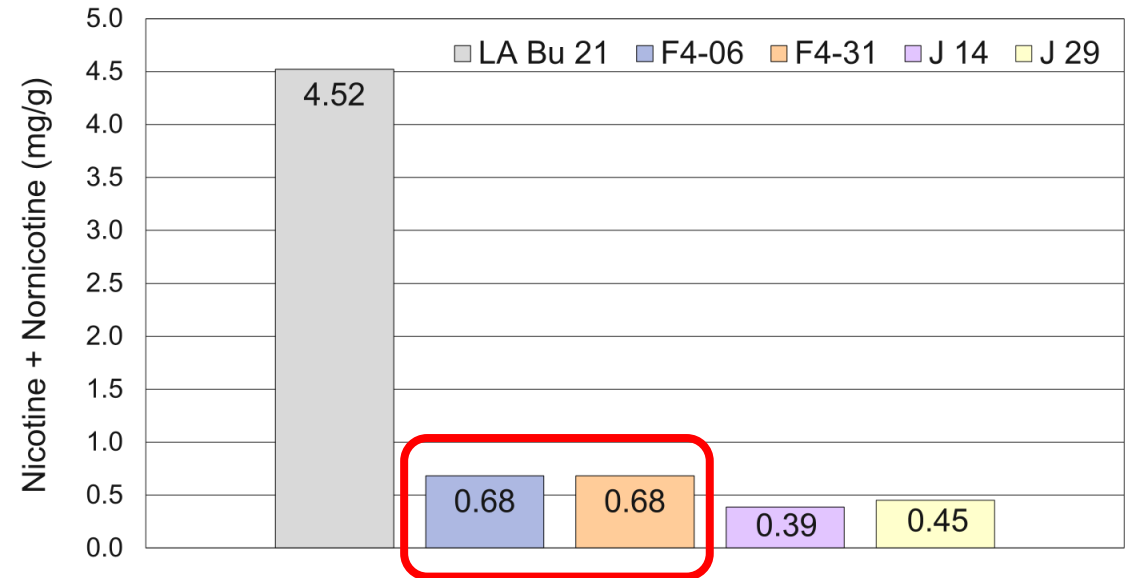


# Novel Gene +LA: Nicotine + Nornicotine Leaf Grade

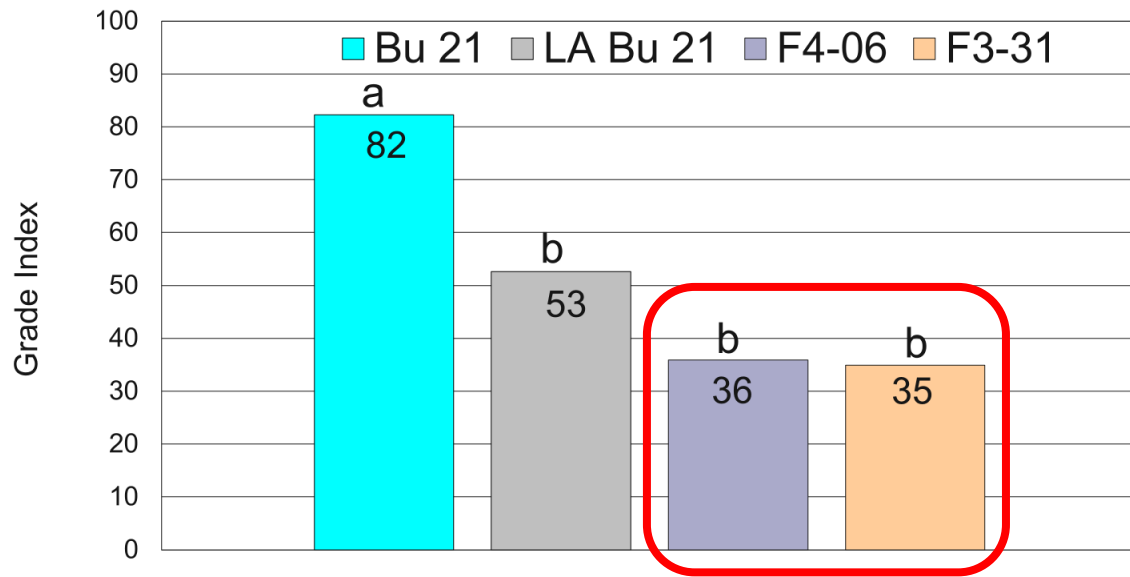
## 2023 Nicotine + Nornicotine Young Plants



## Nicotine + Nornicotine (mg/g) Leaf (B) 2023



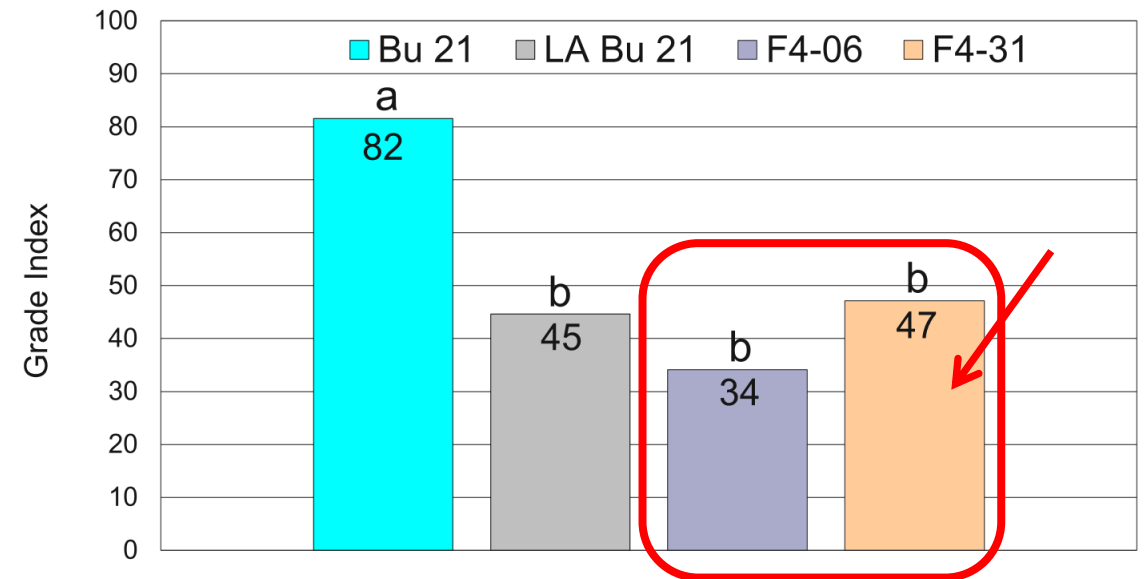
### Grade Index 2022



# Novel Gene +LA: Grade Index

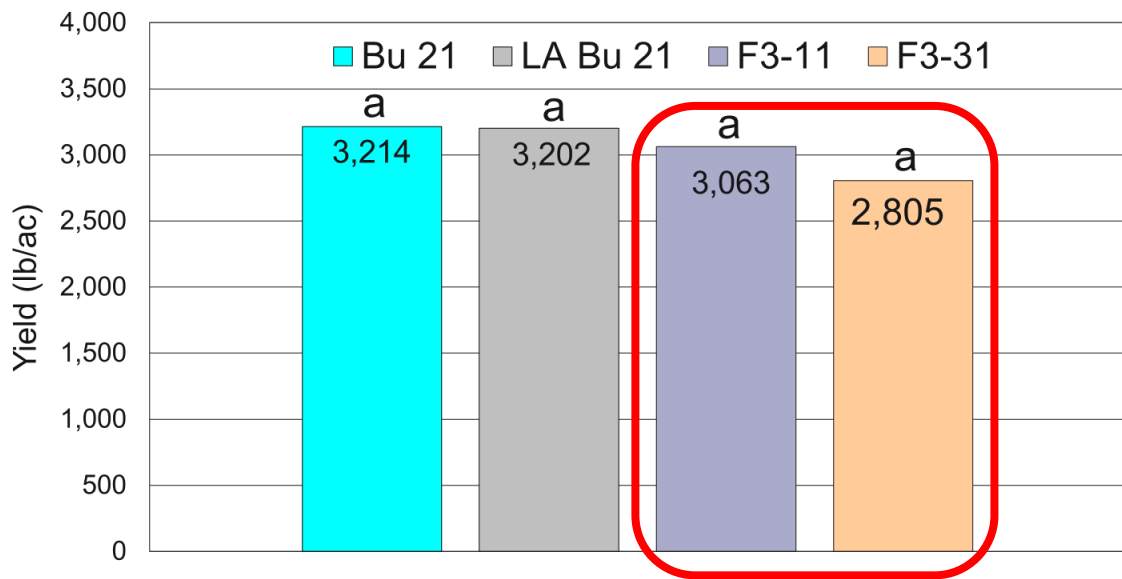


### Grade Index 2023





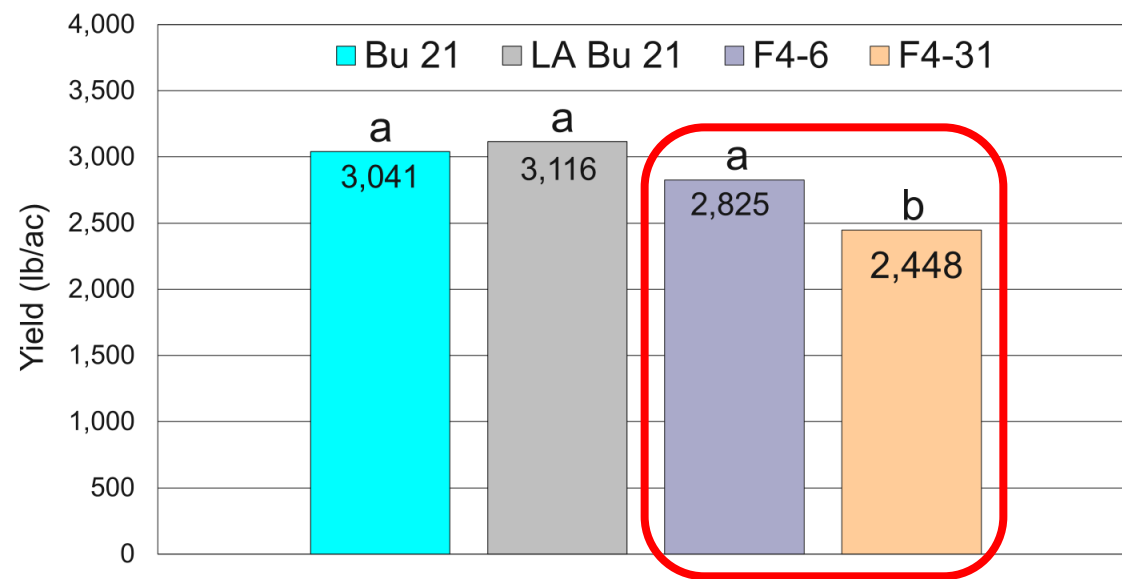
### Yield (lb/ac) 2022



# Novel Gene +LA: Yield



### Yield (lb/ac) 2023





# Novel Gene

## Has potential

- Lower nicotine every year
- Some young F4 lines as low as the gene-edited lines
  - Cured leaf nearly as low
- Quality & possibly yield issues





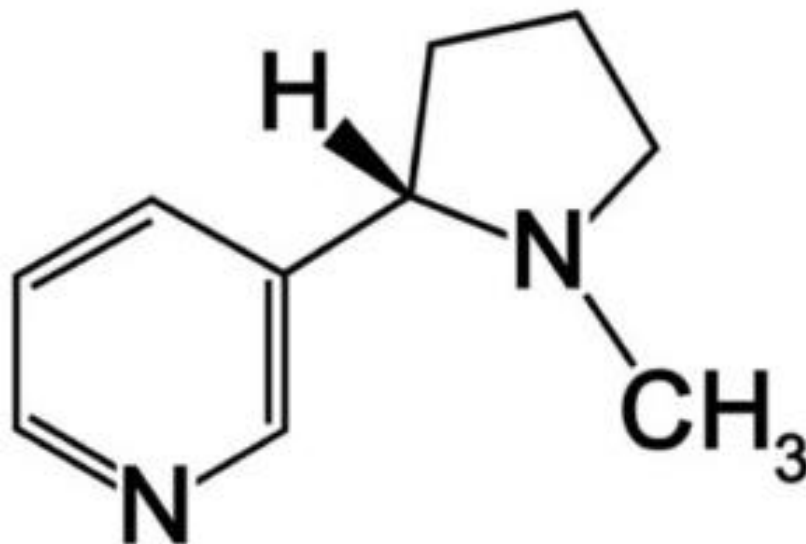


# Can We Meet a 0.5 mg/g Nicotine Limit



**NO**

Not **consistently**, not at this time, not with current knowledge



# Funding

## KTRDC

Funded novel gene work  
Partially funded other work



## PMI

Funded study LA varieties  
+ agronomic practices







[anne.fisher@uky.edu](mailto:anne.fisher@uky.edu)