Final Report to the Council for Burley Tobacco (November 2016)

Title: Evaluation of the Efficacy of HP400 in Reducing TSNAs (2015 season)

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Report type: Final report

Lay Summary: This study was designed to test the efficacy of HP 400, a product reported to reduce TSNAs (Tobacco Specific Nitrosamines) in Europe. Plants were sprayed three days before harvest, with the recommended and double the recommended rate. There were no significant differences between the HP 400 treatments and the controls, for any of the variables. However, TSNAs and alkaloids were generally very low in Kentucky, as a result of excessive early rain. We have found that when TSNAs are low, differences between treatments are often not apparent. It is possible that in a season more conducive to TSNA accumulation, this product may have an effect.

Introduction

Every year, growers waste money on products which do not meet the claims of the manufacturers. Supporting data are needed to advise farmers whether such products are efficacious and economically viable. An effective chemical that would consistently reduce TSNA accumulation would be of great benefit to growers of air-cured tobacco and to the tobacco industry.

HP400 is a natural product tested in Italy and claimed by the manufacturer to reduce TSNAs. According to the label, it is "based on attenuated proteins from microbial fermentation", and "triggers endogenous antioxidant activity". The label further states that "only one application on tobacco may substantially reduce the nitrosamine concentration in the finished product". To date we have been unable to locate any data which substantiate this claim. Previously, we have tested several products claiming to reduce TSNAs and have not found any of them to be efficacious under field conditions. However, it may be possible that an effective treatment does exist, which would be the cheapest, simplest and most reliable way to reduce TSNAs.

The long term objective is to find a product that reduces TSNA accumulation. The short term objective is to test HP400 in the field and evaluate its efficacy in reducing TSNAs in burley tobacco.

Summary of Progress

Procedure – Field Work

Variety

The variety used was TN 90H, a high converter selection of TN 90 which has high TSNA accumulation. The high converter was used because it is easier to detect small differences when TSNA levels are high.

Label recommendations for TSNA program
120 g/ha or 48.6 g/acre product
800-900 L/ha or 85-96 gallons/acre water
Apply 7-3 days before harvest

Treatments

The treatments were two controls (water control and unsprayed) and two rates of HP400; the recommended rate and double the recommended rate, as specified on the label (Figure 1). Both rates of HP400 were applied with a backpack sprayer in 90 gallons/acre, 49 ml per plant water (Figure 2), three days before harvest. The water control was applied with a backpack sprayer at the rate of 50 gallons/acre, 27 ml plant, one day before harvest. The rates for this product are low compared with the rates of most agrochemicals; 1.7 and 3.4 oz/acre.

1. No spray (unsprayed control)
2. Water spray (solvent control), 50 gallons/acre, 1 day before harvest
3. HP400 recommended rate, 48.6 g/acre product in 90 gallons/acre water, 3 days before harvest
   1.7 oz/acre of product
4. HP400 double rate, 97.2 g/acre product in 90 gallons/acre water, 3 days before harvest
   3.4 oz/acre of product

Design

The design was four randomized complete blocks with four spray treatments and appropriate border rows, with some blocking for type of spray (HP 400 and checks).

Agronomic details

The tobacco was grown with all normal recommended practices. Float trays were seeded March 24th, and the study was transplanted May 28th. Six days before transplanting, we applied 200 lb/ac N as urea, and 350 lb/ac K_2O as potassium sulfate. The herbicides sulfentrazone (Spartan) and clomazone (Command) were applied pre-emergent immediately before transplanting. Planting water chemicals were mefenoxam (Ridomil), imidacloprid (Admire) and chlorantraniliprole (Coragen).

The early part of the season was very wet; there was a heavy rainstorm the day of transplanting and for the next 17 days, it was too wet to get into the field. Rainfall was 1¾ inches in the last week of May, 10 inches in June and 14 inches in July. As a result of this excessive early rain, roots did not develop well, and the root systems were small. The last part of the season was much drier, with only 3¼ inches of rain in August and long dry spells. Because of its small root system, the crop did not tolerate the dry conditions well, and there was considerable firing at the bottom of the plant.

We had an unusual spectrum of pests and diseases, related largely to the wet weather. There was target spot at the bottom of the plant, which has been a common occurrence for the last few years. However, there was a considerable amount of angular leaf spot, which is unusual for Kentucky. There was also a heavy infestation of Japanese beetles (Figure 3); this is unusual as they are considered a minor pest in Kentucky.

The first flowers were counted (pink flowers, not open flowers) July 22nd (6%). The study was topped July 27th, with 35% pink flowers. Four days before topping (July 23rd), we applied 50% fatty alcohol

suckeride (Offshoot T), and the insecticides thiamethoxam (Actara) and chlorantraniliprole (Coragen). Immediately after topping, we applied the suckerides maleic hydrazide (MH), Butralin (Butralin) and 50% fatty alcohol (Offshoot T). Suckers were very small at this stage, and sucker control was excellent.

The HP400 sprays were applied with a backpack sprayer (Figure 2) three days before harvest, August 24th, and the water control was applied the day before harvest, August 26th (see *Treatments* for details). The study was harvested 31 days after topping, on August 27th. Thirty plants were harvested for each plot; five sticks of six plants each. The tobacco was left sticked out in the field until the next day, when it was picked up and put onto a rail wagon (Figure 4) which was parked in the barn until housing four days after harvest, on August 31st (Figure 5).

The tobacco was taken down in January and sampled for chemical analysis.

Sampling and sample preparation for chemical analysis

At stripping, only the inner four plants on each of five sticks were sampled; the outer two plants were discarded. The fourth leaf from the top of the plant was sampled; bulk samples of 20 leaves per plot. Leaves were stemmed, air-dried and both lamina and midrib were ground to pass through a 1 mm screen.

Statistical analysis

PROC MIXED of SAS 9.1 (SAS Institute, Cary, NC, USA) was used for an analysis of variance appropriate for a complete randomized block design with blocking. The analysis used four independent treatments, but blocked on the 'chemical' factor.

The residuals were visually checked for heteroscedasticity and transformation of the data was found to be necessary for some variables, in order to conform to the assumption of equal variance. Natural logarithmic or exponential transformations were done where necessary (Table 1), prior to means separation procedures. Means were separated according to protected Fisher's least significant difference.

<u>Procedure – Analytical Laboratory</u>

Constituents analyzed

Both lamina and midrib were analyzed for all constituents.

TSNAs: individual TSNAs and total TSNAs (date are not presented for NNK and NAB, because the levels were very low

Alkaloids: individual alkaloids, total alkaloids, conversion (data are not presented for individual alkaloids)

Nitrate nitrogen

Nitrite nitrogen

Total nitrogen

Laboratory analysis

TSNA analyses were run in our laboratory using gas chromatography with TEA (Thermal Energy Analyzer) chemiluminescence detection and methylene chloride extraction, and alkaloid analyses were done on a GC (gas chromatogram) with FID (flame ionization detection).

Nitrate nitrogen and nitrite nitrogen were measured colormetrically with Griess reagent. Nitrate was reduced quantitatively to nitrite with a copperized cadmium reductor in microplate wells and Griess reagent added for colorimetric measurement at 542 nm. Total nitrogen was measured using the Kjeldahl method.

Results and Discussion

TSNAs and alkaloids were unusually low in Kentucky in 2015, as a result of the heavy early rain and consequent small root systems. Total TSNAs for the high converter TN 90H are typically over 10 ppm, but in the last five years, we have measured TSNAs over 10 ppm only once, in 2012 (Figure 6). Figure 6 shows total TSNAs for the TN 90H check treatment in studies transplanted in the last week of May from 2011 to 2015 (the 2014 crop was destroyed by hail). Total TSNAs in 2015 for these studies were below 2 ppm, which is unprecedented for TN 90H – these values would be more typical of the low converter, TN 90LC. Leaf nitrate in 2015 was also very low; lamina nitrate nitrogen levels below 800 ppm and midrib nitrate nitrogen levels below 5,000 ppm are unprecedented (Figures 9C, 9D). Past experience has shown us that when TSNAs are very low, it is very difficult to detect treatment differences.

There were no significant differences between treatments for any of the variables (Table 1, Figures 7-9). One might speculate that in a season more conducive to TSNA accumulation, HP 400 might have had a significant impact on reducing TSNAs. However, the *p* values in the ANOVA table were very high (Table 1), and there was no trend to lower TSNAs with the HP 400 treatment (Figure 7).

Conclusions

In this one study, HP 400 did not reduce TSNAs. However, it was a season very unfavorable for TSNA accumulation, and it is possible that in a more typical season, this product may be efficacious.

Plans for Future Work

This study was repeated in 2016, but we do not yet have any results. In view of the very unsatisfactory results from 2015, we would like to repeat the study in 2017.

Figures and Tables

Table 1: Effect of HP400 sprays on all variables: ANOVA *p* values and transformations

Constituent	Lamina Midrib	Transformation	*p* Value	Significance
NNN	Lamina	None	0.50	NS
NNN	Midrib	Log	0.48	NS
NAT	Lamina	None	0.37	NS
NAT	Midrib	Log	0.45	NS
Total TSNAs	Lamina	None	0.47	NS
Total TSNAs	Midrib	Log	0.46	NS
Conversion	Lamina	None	0.12	NS
Conversion	Midrib	None	0.14	NS
Total Alkaloids	Lamina	None	0.12	NS
Total Alkaloids	Midrib	None	0.20	NS
NO_2 N	Lamina	Log	0.11	NS
NO_2 N	Midrib	None	0.16	NS
NO_3 N	Lamina	None	0.67	NS
NO_3 N	Midrib	None	0.68	NS
Total N	Lamina	Exponential	0.82	NS
Total N	Midrib	None	0.70	NS

NS = not significant (p>0.05)

Figure 1: HP 400 label

Figure 2: Spray application with a backpack sprayer

Figure 3: Japanese beetles

Figure 4: Railwagon after harvest

Figure 5: Barn prior to housing

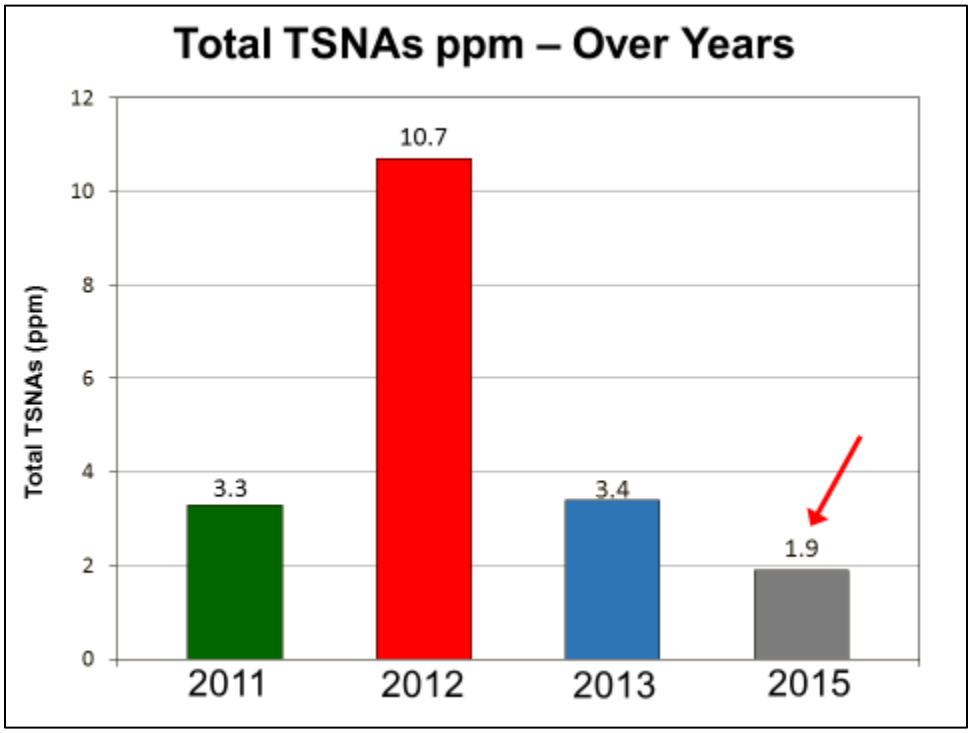
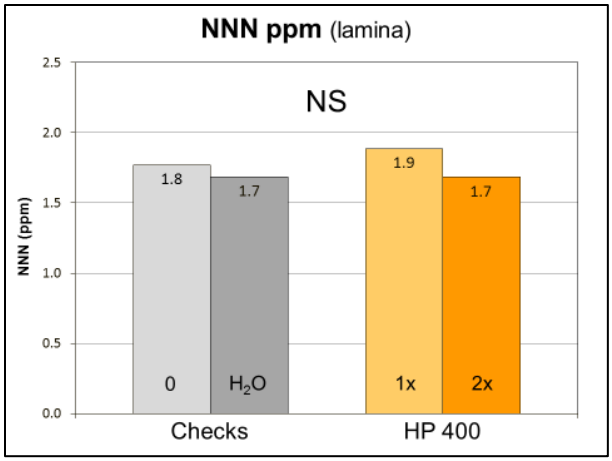


Figure 6: Total TSNAs in TN 90H transplanted in the last week of May, for the four years 2011 – 2015



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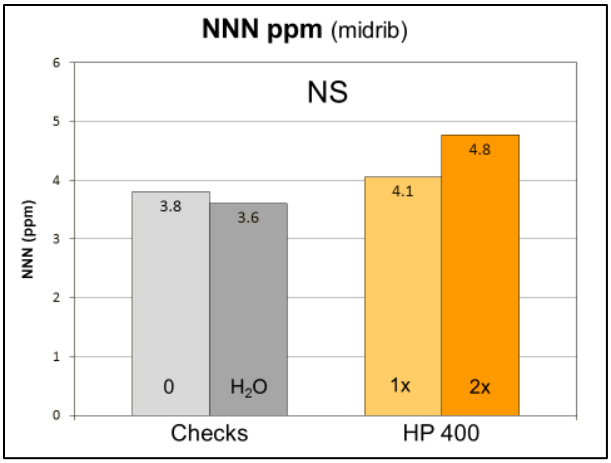

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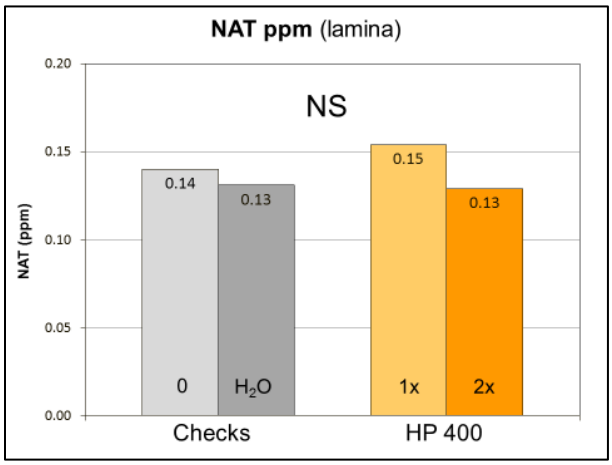

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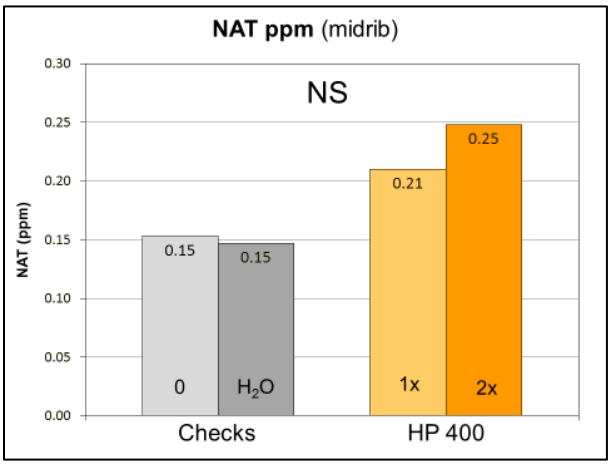

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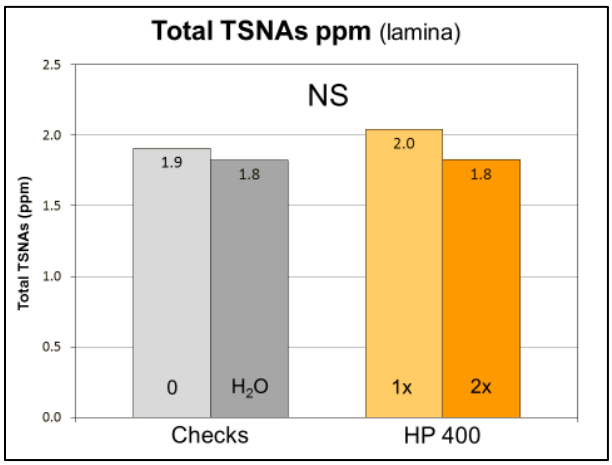

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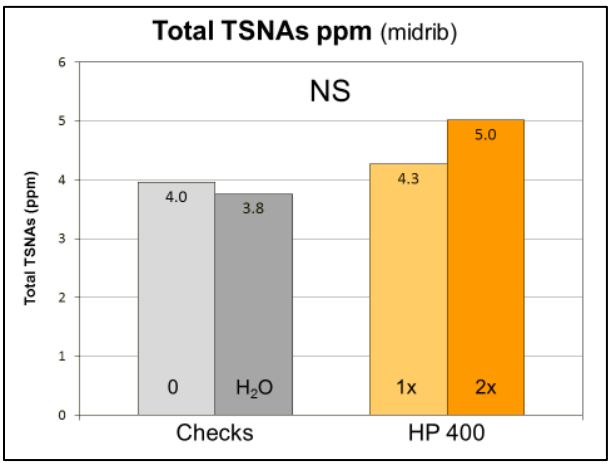
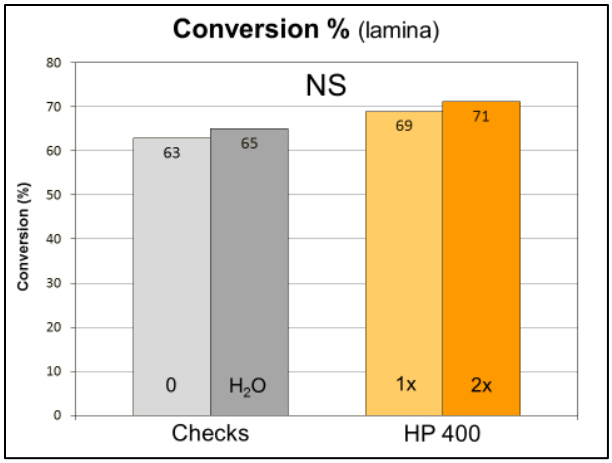


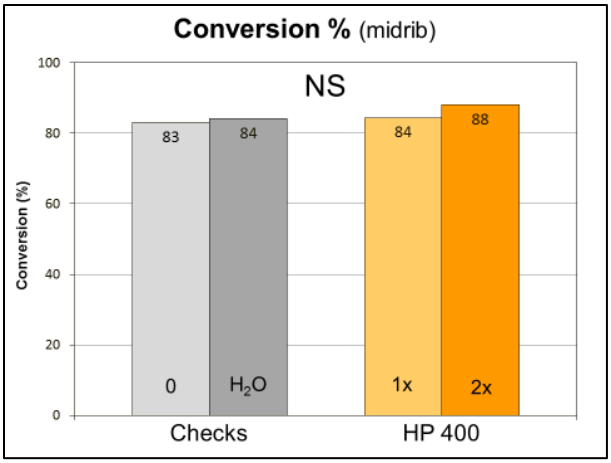
F

Figure 7: Effect of HP400 sprays on TSNAs **A.** Lamina NNN **B.** Midrib NNN **C.** Lamina NAT **D.** Midrib NAT **E.** Lamina Total TSNAs **F.** Midrib Total TSNAs

NS = not significant (p>0.05)



A



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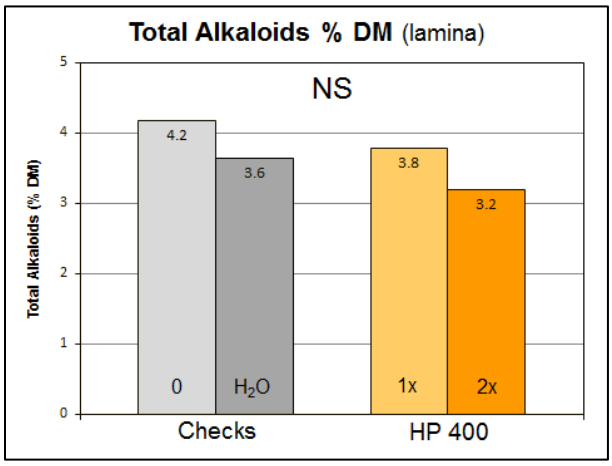

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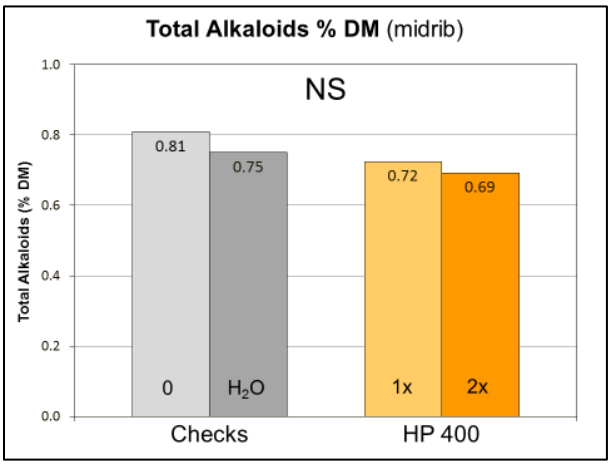
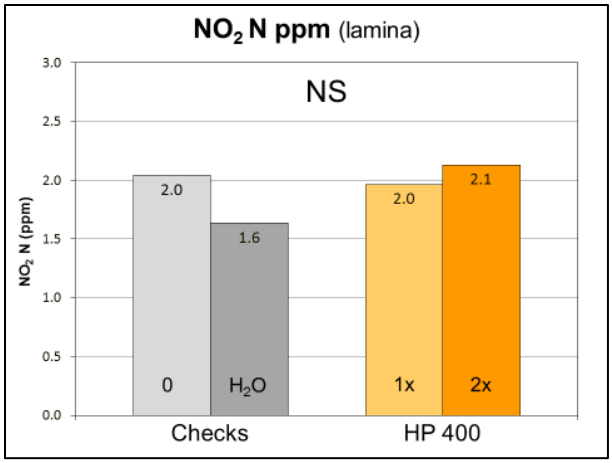


D

Figure 8: Effect of HP400 sprays on alkaloids **A.** Lamina Conversion **B.** Midrib Conversion **C.** Lamina Total Alkaloids **D.** Midrib Total Alkaloids

NS = not significant (p>0.05)



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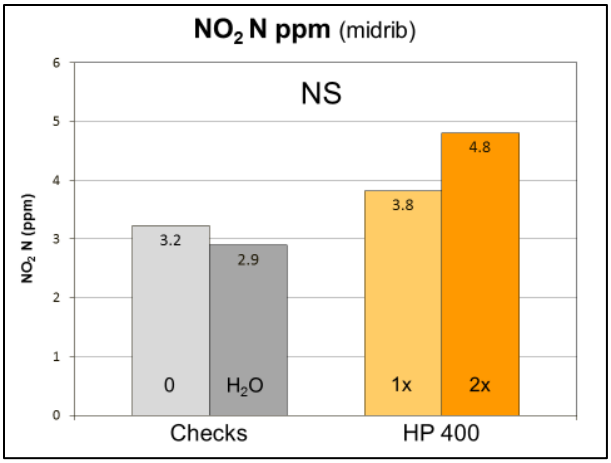

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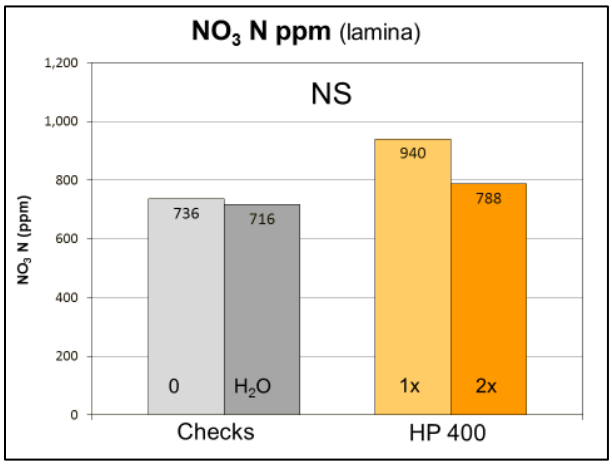

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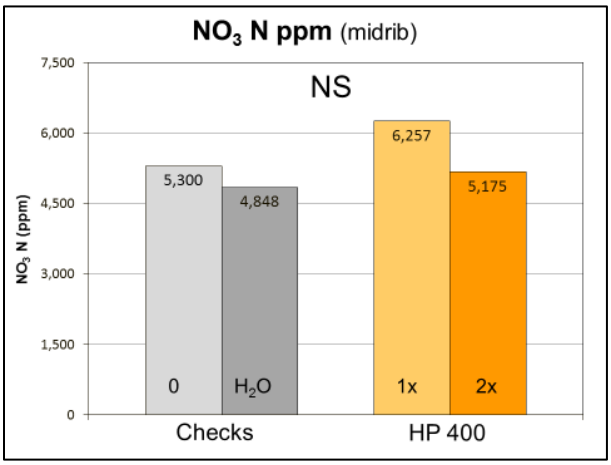

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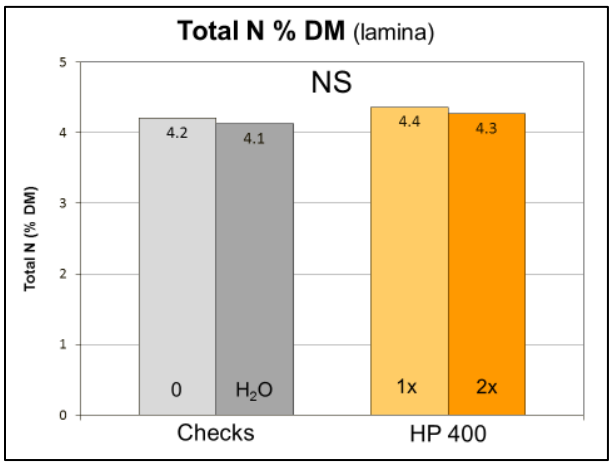

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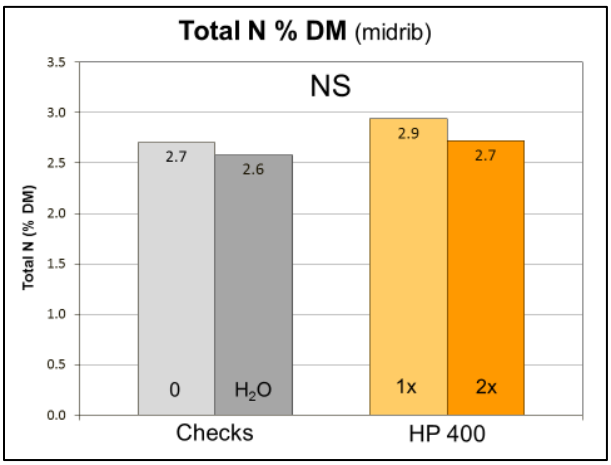

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Figure 9: Effect of HP400 sprays on nitrogenous constituents **A.** Lamina NO_2 N **B.** Midrib NO_2 N **C.** Lamina NO_3 N **D.** Midrib NO_3 N **E.** Lamina Total Nitrogen **F.** Midrib Total Nitrogen

NS = not significant (p>0.05)