Final Report to the Council for Burley Tobacco (October 2017)

Title: Evaluation of the Efficacy of HP400 in Reducing TSNAs (2015, 2016 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 TSNAs. However, TSNAs, alkaloids and nitrate nitrogen were generally very low in Kentucky in 2015, as a result of excessive early rain. We have found that when TSNAs are low, differences between treatments are often not apparent. TSNAs were higher in 2016, but alkaloids and conversion were lower than they should have been, because of contaminated seed received from an outside source. The only variable significantly impacted by the HP 400 treatments was conversion. In some cases, HP 400 may have increased conversion, but this effect was not entirely consistent.

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

<u>Procedure – Field Work</u>

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 in 90 gallons/acre, 49 ml per plant water three days before harvest with a backpack sprayer in 2015, and with a high clearance tractor in 2016 (Figure 5). The water control was applied 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.

Agronomic details 2015

The tobacco was grown with all normal recommended practices. Float trays were seeded March 24th, and the study was transplanted May 28th (Figure 2). 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 4); 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 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 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 27t (Figure 6). 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 7) which was parked in the barn until housing four days after harvest, on August 31st.

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

Agronomic details 2016

The tobacco was grown with all normal recommended practices, except that we used a higher rate of nitrogen than usual (300 lb/acre N as urea, instead of the recommended 200 lb/acre). We did this in an attempt to get higher levels of TSNAs, because in the last few years, TSNAs have been so low that most treatment differences were non-significant. Lime was applied to the field at the rate of 3 tons/acre. Float trays were seeded March 28th, and the study was transplanted May 31st (Figure 2). Just before transplanting, we applied 300 lb/acre N as urea, and 270 lb/acre 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 rainfall in the early part of the season was ideal, but dried up during the grand growth stage. July was so dry that we applied drip irrigation on July 20th, almost two weeks before topping (Figure 3).

As in 2015, we had an unusual spectrum of pests and diseases. There was a heavy infestation of Japanese beetles (Figure 4); this is unusual as they are considered a minor pest in Kentucky. We sprayed to control them with thiamethoxam (Actara) two weeks before topping (July 19th). There was target spot at the bottom of the plant, which has been a common occurrence for the last few years, necessitating spraying with azoxystrobin (Quadris) a week before topping, on July 27th.

The first flowers were counted (pink flowers, not open flowers) July 27th (18%). The study was topped five days later (August 1st), nine weeks after transplanting. Two days after topping, we applied fatty alcohol (Offshoot T), maleic hydrazide (MH) and butralin (Butralin).

The HP400 sprays were applied three days before harvest, August 29th, and the water control was applied the day before harvest, August 31st (see *Treatments* for details). We used a high clearance tractor (Figure 5), unlike 2015, when we applied the treatments with a backpack sprayer. The study was harvested 31 days after topping, on September 1st (Figure 6). 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 7) which was parked in the barn until housing five days after harvest, on September 6th.

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.

<u>Procedure – Analytical Laboratory</u>

Constituents analyzed

Both lamina and midrib were analyzed for all constituents.

TSNAs: individual TSNAs and total TSNAs (data are not presented for 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.

<u>Procedure – Statistical Analysis</u>

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. Data were analyzed for each year (2015 and 2016) separately and for the years combined. The across-years model included a random factor for year (thereby accounting for the fact that the overall level of each response could be different in each year), and a rep*year interaction, to ensure that rep 1 in 2015 and rep 1 in 2016 are not considered the same rep.

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, log-log 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>Results and Discussion</u>

TN 90H seed

We have ascertained that the TN 90H seed used in 2016 was contaminated with a low alkaloid variety. Data from a two-year study (2015, 2016) incorporating both TN 90LC and TN 90H, are presented in Figures 8A–8D. The same seedlot of TN 90LC, sourced from a commercial seed company, was used in both years.

The TN 90H seed used in the 2015 study was produced by us in 2008. In 2014, new seed was produced for us by an outside source; this seed was used in 2015 because the 2008 seed was six years old and losing vigor. Total alkaloids (TAs) in 2015 were generally lower than in 2016, as shown for TN 90LC: 3.2% DM and 4.3% DM, respectively (Figure 8A). Conversion for TN 90LC in both years was consistent with that expected for a low converter variety (Figure 8B). TAs are generally slightly lower in TN 90H than in TN 90LC, because some alkaloids are lost or further metabolized in the conversion process. The TN 90H TAs in 2015 were 2.5% DM, consistent with expectation (Figure 8C). However, the TN 90H TAs in 2016 were very much lower than expected (1.0% DM). Conversion in 2016 was also much lower than expected; 36% instead of the usual 70-80% (Figure 8D). We suspected a seed mixture in 2016, as some of the plants did not look true to type, so we grew out both seedlots in 2017 and sampled individual young plants (Figures 8E, 8F). All plants grown from our 2008 seedlot (used in 2015) had >90% conversion and (nicotine + nornicotine) about 1% DM (green oval, Figure 8E), as would be expected for TN 90H. However, the 2014 seedlot (used in 2016) was clearly a mixture of TN 90H, with >90% conversion and (nicotine + nornicotine) around 1% DM (green oval, Figure 8F), and a low alkaloid, low converter line with mostly <10% conversion and (nicotine + nornicotine) all around 0.1% DM (red oval, Figure 8F). This seed was produced in 2014, and the adjacent seed plot was LA Burley 21 – this is a low alkaloid, low converter line. It seems that the 2014 seed is a mixture of about 70% LA Burley 21 and 30% TN 90H. This has profound implications for the TSNAs measured in the 2016 trials, because both conversion and alkaloids are much lower than they should be, and both have a very significant impact on TSNA accumulation.

2015

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 lamina total TSNAs in 2105 (Figure 12A) were <2 ppm. This 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 16A, 16B). Past experience has shown us that when TSNAs are very low, it is very difficult to detect treatment differences.

2016

2016 was generally more favorable for TSNA accumulation than 2015; alkaloids and nitrates were higher, and TSNAs were higher. However, because of the seed mixture described above, conversion and total alkaloids in this study were lower in 2016. Despite this, TSNAs were still higher than in 2015.

TSNAs

All TSNAs were higher in 2016 than in 2015: lamina total TSNAs ranged from 1.8-2.0 ppm in 2015 and 1.9-3.1 ppm in 2016 (Figure 12). If the correct TN 90H seed had been used, TSNAs would certainly have been even higher.

There were no significant differences between HP 400 treatments and checks for any of the TSNAs (Table 1, Figures 9-12).

Alkaloids

Both conversion and total alkaloids were lower in 2016 than in 2015 (although alkaloids generally were higher in 2016 than in 2015), because of the seed mixture (Figures 13, 14).

The general, but not wholly consistent, trend in conversion was an increase in the HP 400 treatments (Table 1, Figure 13). In 2015, lamina conversion in the high HP 400 rate was significantly higher than in both checks, and lamina conversion in the low HP 400 rate was higher than in the unsprayed check (Figure 13A). Midrib conversion was higher in the high HP 400 rate than in both checks (Figure 13B). There were no significant differences between treatments in 2016 (Figures 13C, 13D, Table 1). In the years combined, lamina conversion in both HP 400 rates was higher than in the unsprayed check (Figure 13E). Midrib conversion in the high HP 400 rate was higher than in the unsprayed check (Figure13F).

The only significant difference in total alkaloids was in the 2015 lamina (Table 1, Figure 14). Lamina total alkaloids in the high rate of HP 400 were lower than in the unsprayed check. While this difference was statistically significant in the 2015 lamina, it was not consistent across years and tissue type. There is no physiological explanation for this, as alkaloids are accumulated by the time of harvest: it is highly unlikely that any spray a few days before harvest would have any effect, and certainly once alkaloids are accumulated, they cannot be decreased.

Nitrogenous constituents

Lamina nitrite nitrogen was very low in both years; in the midrib it was higher in 2016 than in 2015; maximum 7.7 ppm v. 4.8 ppm (Figure 15). The only significant difference was in the 2015 lamina (Table 1), where both rates of HP 400 were higher than the water check (Figure 15A). However, the nitrite levels were too low (1.6-2.1 ppm) for this difference to be biologically significant.

Nitrate nitrogen was unusually low in 2015, but was at normal levels in 2016 (716-940 ppm lamina 2015, 4,269-5,245 ppm lamina 2016, Figures 16A, 16B). There were no significant differences between the HP400 treatments and checks (Table 1, Figure 16).

Total nitrogen (total N) in the lamina was similar in the two years (4.1-4.5% DM), but in the midrib, it was lower in 2015: 2.6-2.9% DM vs. 4.2-4.5% DM (Figure 17). There were no significant differences between the HP400 treatments and checks (Table 1, Figure 17).

<u>Conclusions</u>

In this two-year study, HP 400 did not reduce TSNAs. One might speculate that in conditions 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 consistent trend to lower TSNAs with the HP 400 treatments (Figures 9-12).

Plans for Future Work

This study is being repeated in 2017, with new seed produced and tested by us.

Figures and Tables

Table 1: Effect of HP 400 sprays on all variables: ANOVA *p* values and transformations

		2015			2016			Years Combined		
Constituent	**Lamina Midrib**	**Transformation**	***p* Value**	**Significance**	**Transformation**	***p* Value**	**Significance**	**Transformation**	***p* Value**	**Significance**
NNN	Lamina	log	0.466	NS	log	0.944	NS	log	0.922	NS
NNN	Midrib	log	0.443	NS	log	0.996	NS	log	0.993	NS
NAT	Lamina	log	0.329	NS	log	0.928	NS	log-log	0.929	NS
NAT	Midrib	log	0.403	NS	log	0.983	NS	log	0.817	NS
NNK	Lamina	log	0.272	NS	log	0.814	NS	log	0.404	NS
NNK	Midrib	log	BDL	NS	log	0.540	NS	log	0.425	NS
Total TSNAs	Lamina	log	0.445	NS	log	0.946	NS	log	0.933	NS
Total TSNAs	Midrib	log	0.416	NS	log	0.999	NS	log	0.987	NS
Conversion	Lamina	none	0.0318	*	none	0.308	NS	none	0.0241	*
Conversion	Midrib	none	0.0432	*	none	0.465	NS	exponential	0.0262	*
Total Alkaloids	Lamina	none	0.0323	*	none	0.676	NS	none	0.858	NS
Total Alkaloids	Midrib	none	0.0831	NS	none	0.581	NS	none	0.850	NS
NO_2 N	Lamina	log	0.0273	*	log	0.926	NS	none	0.690	NS
NO_2 N	Midrib	log	0.0908	NS	log	0.831	NS	log-log	0.660	NS
NO_3 N	Lamina	none	0.647	NS	none	0.393	NS	log	0.505	NS
NO_3 N	Midrib	none	0.657	NS	none	0.385	NS	none	0.424	NS
Total N	Lamina	none	0.750	NS	none	0.246	NS	none	0.313	NS
Total N	Midrib	none	0.685	NS	none	0.524	NS	none	0.440	NS

[a] = below detectable limit

NS = not significant (*p*>0.05)   * = significant (*p*>0.05)

Figure 1: HP 400 label

Figure 2: Transplanting

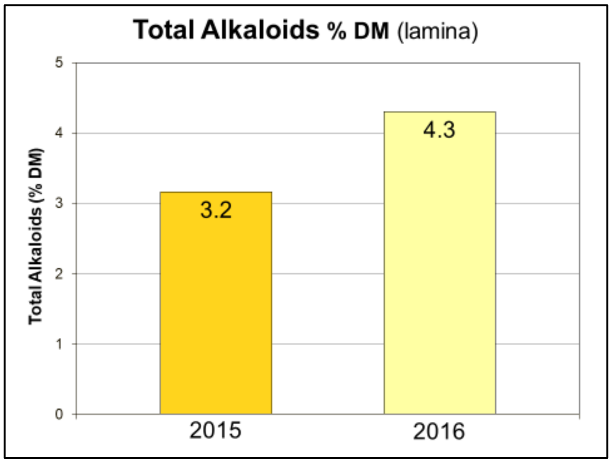
Figure 3: Drip irrigation

Figure 4: Japanese beetles

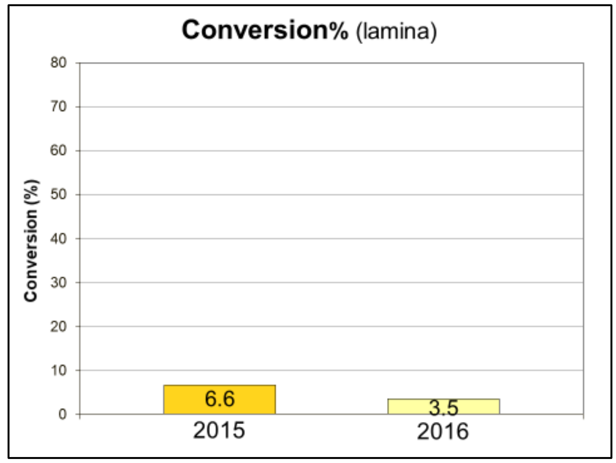
Figure 5: Spray application with a high clearance tractor

Figure 6: Harvesting

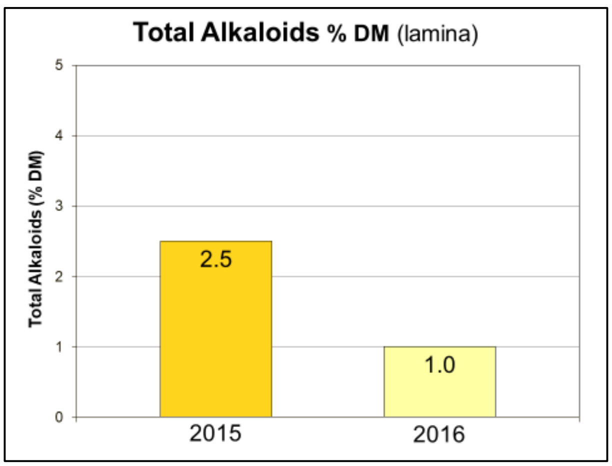
Figure 7: Railwagon after harvest



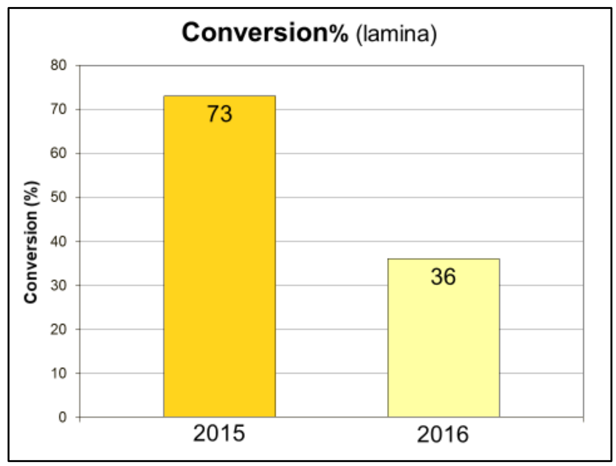
A. TN 90LC



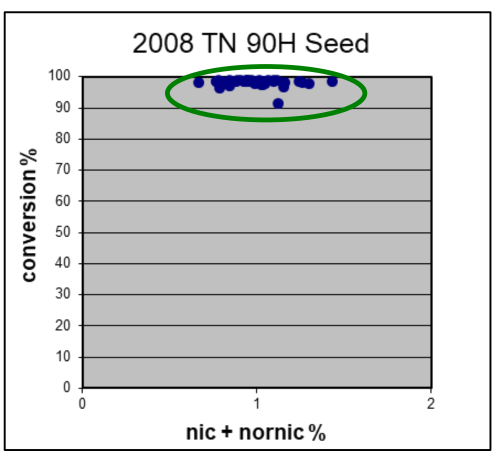
B. TN 90LC



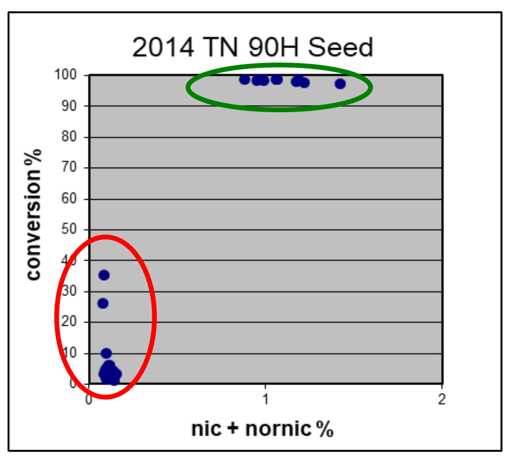
C. TN 90H



D. TN 90H

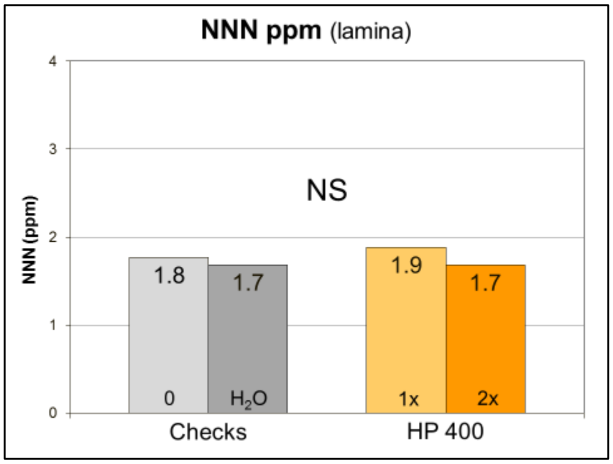


E. 2008 seedlot, used in 2015

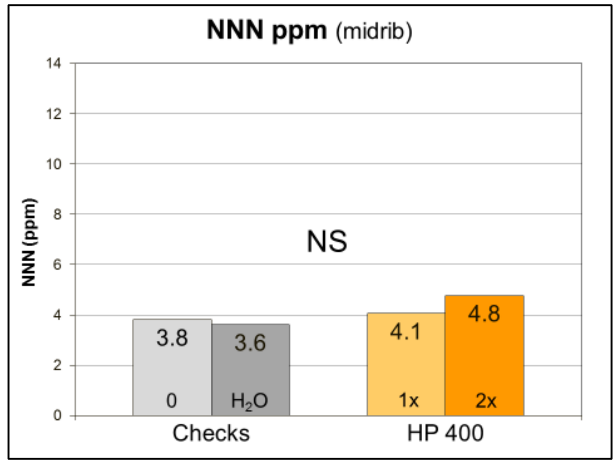


F. 2014 seedlot, used in 2016

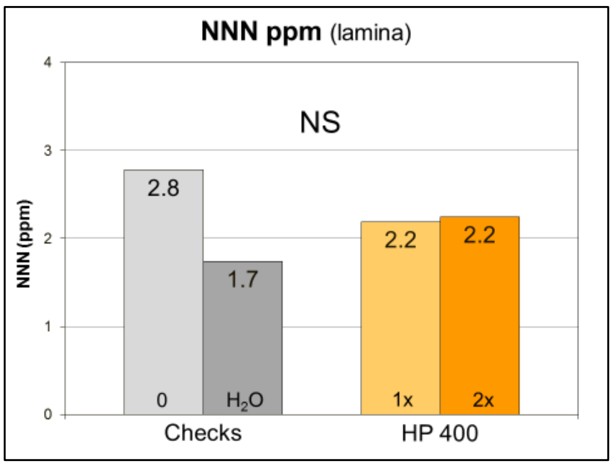
Figure 8: Total alkaloids and conversion in TN 90H and TN 90LC, 2015 and 2016, showing the difference between TN 90H seedlots used in 2015 and 2016 (data from a different study). **A.** TN 90LC, lamina total alkaloids (TA) **B.** TN 90LC, lamina conversion **C.** TN 90H, lamina total alkaloids (TA) **D.** TN 90H, lamina conversion **E, F.** Scatter diagrams for individual TN 90H plants, two seedlots



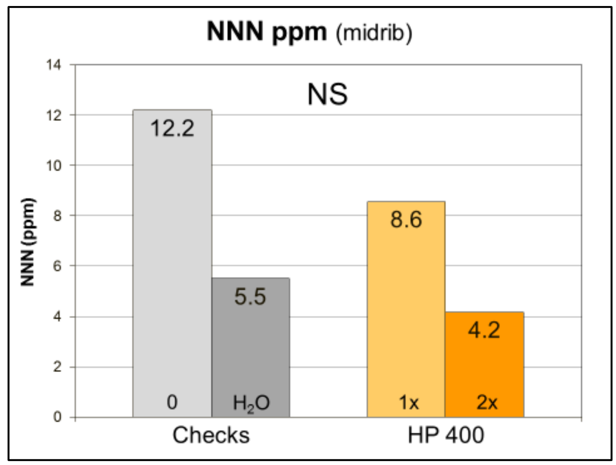
A. 2015



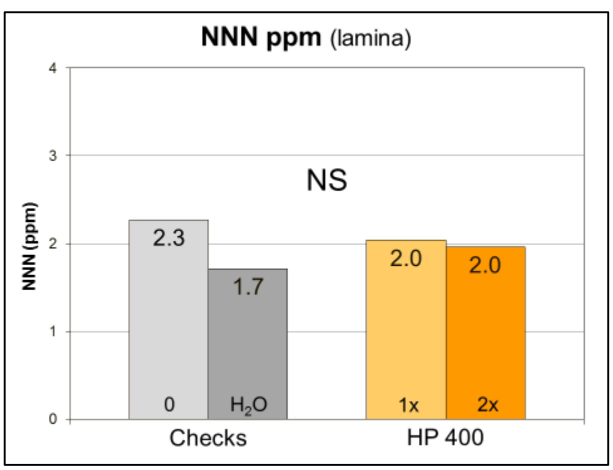
B. 2015



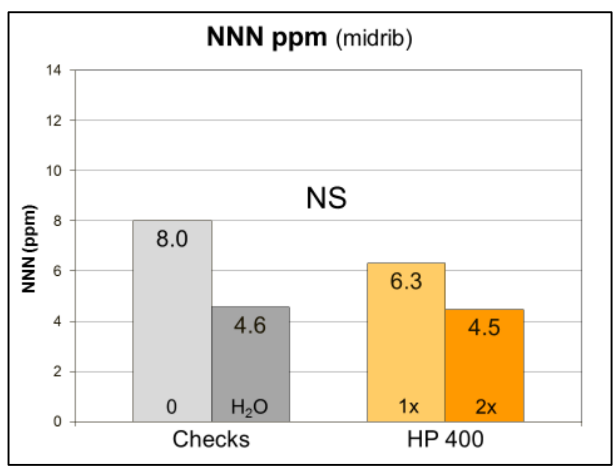
C. 2016



D. 2016



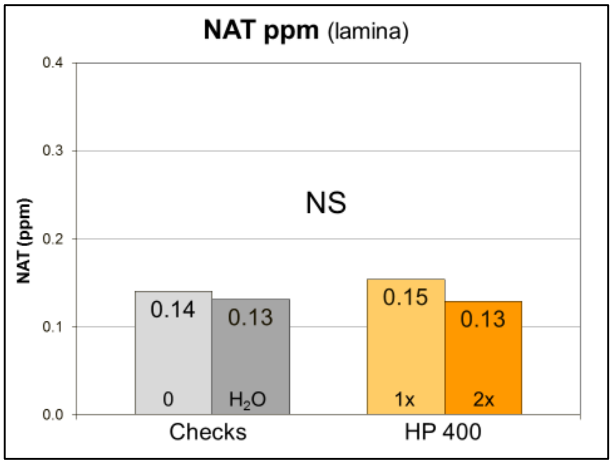
E. Years combined



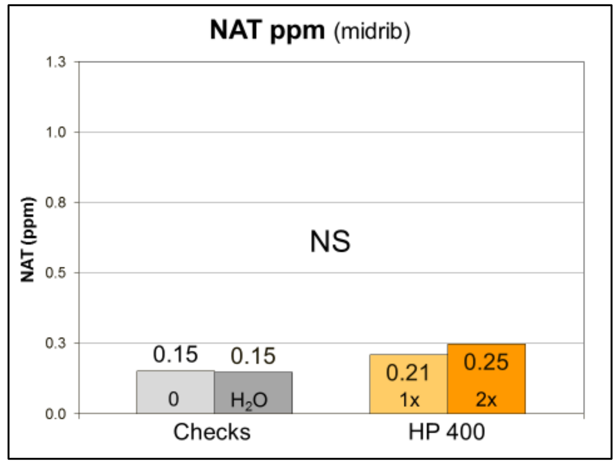
F. Years combined

Figure 9: Effect of HP 400 sprays on NNN. **A.** Lamina NNN, 2015 **B.** Midrib NNN, 2015 **C.** Lamina NNN, 2016 **D.** Midrib NNN, 2016 **E.** Lamina NNN, years combined **F.** Midrib NNN, years combined

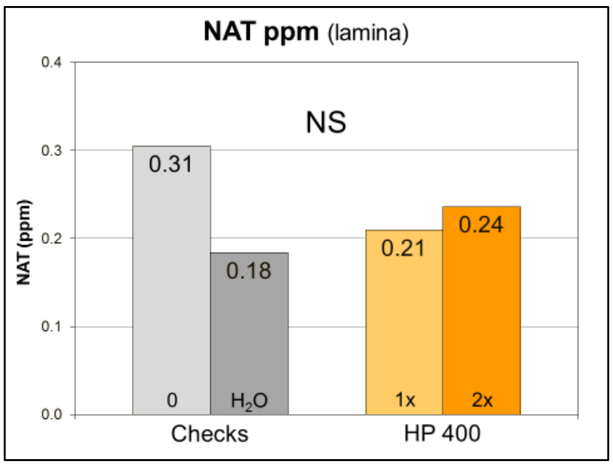
NS = not significant (p>0.05)



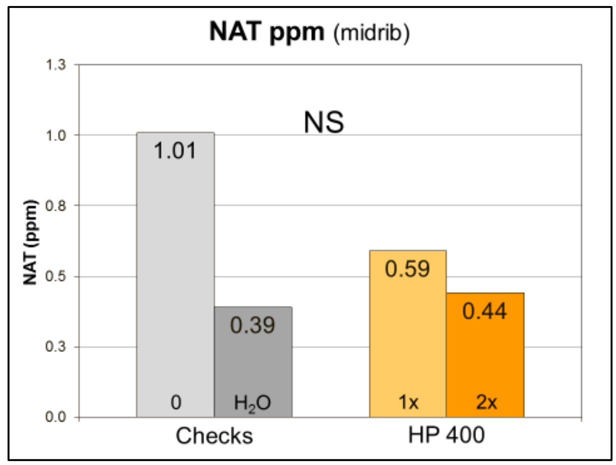
A. 2015



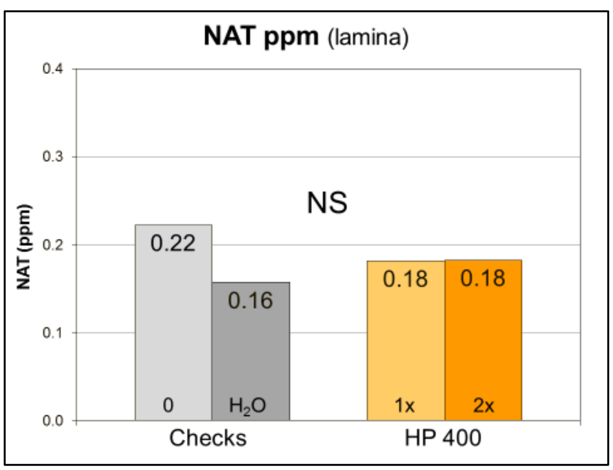
B. 2015



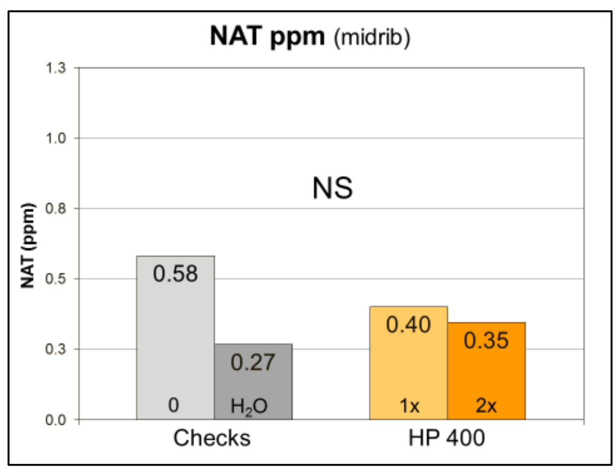
C. 2016



D. 2016



E. Years combined



F. Years combined

Figure 10: Effect of HP 400 sprays on NAT. **A.** Lamina NAT, 2015 **B.** Midrib NAT, 2015 **C.** Lamina NAT, 2016 **D.** Midrib NAT, 2016 **E.** Lamina NAT, years combined **F.** Midrib NAT, years combined

NS = not significant (p>0.05)

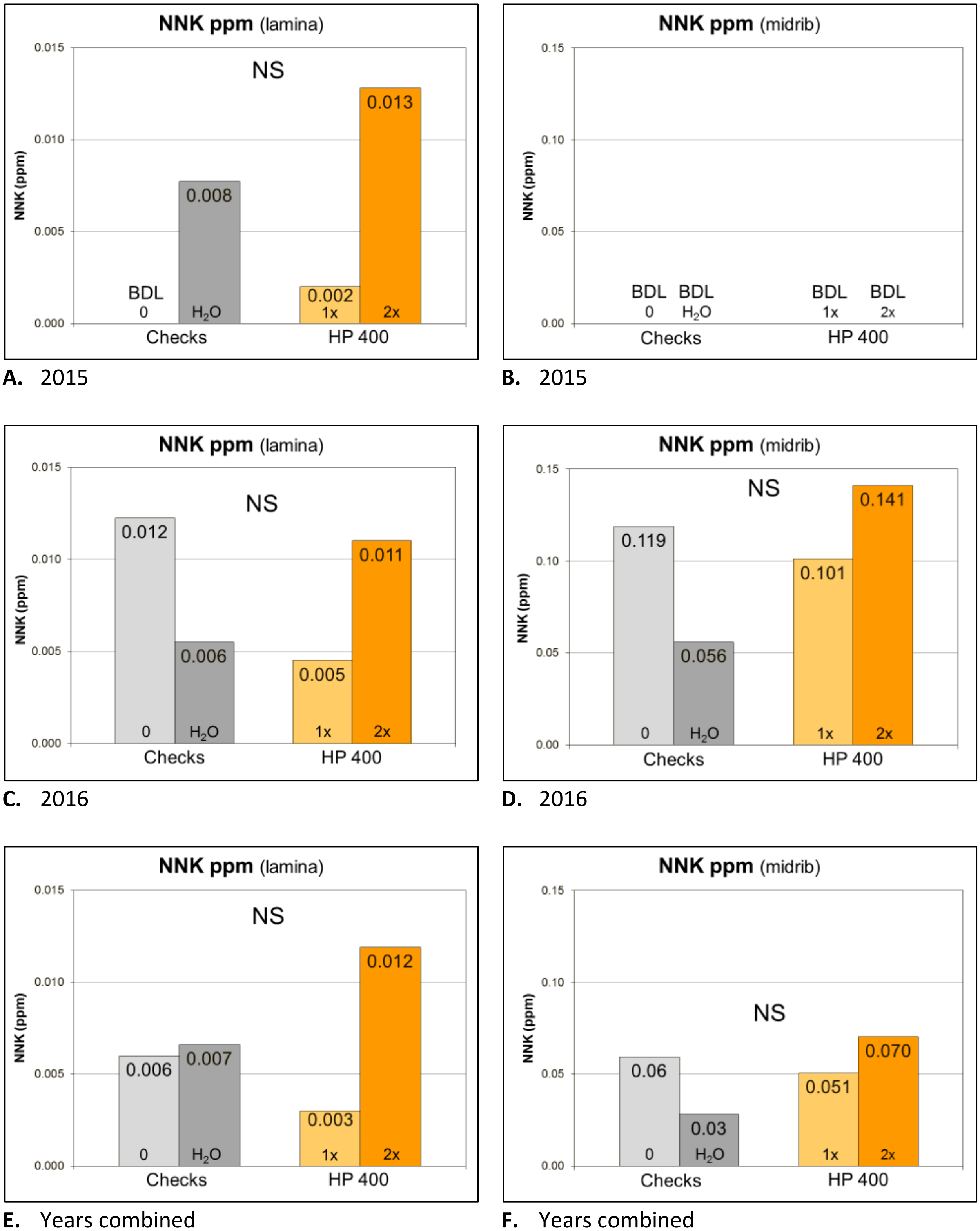
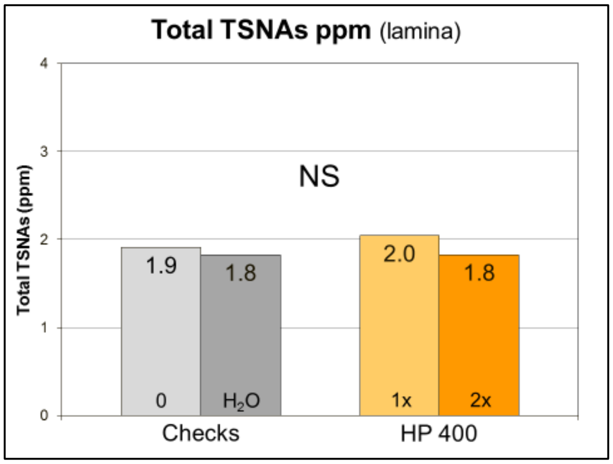
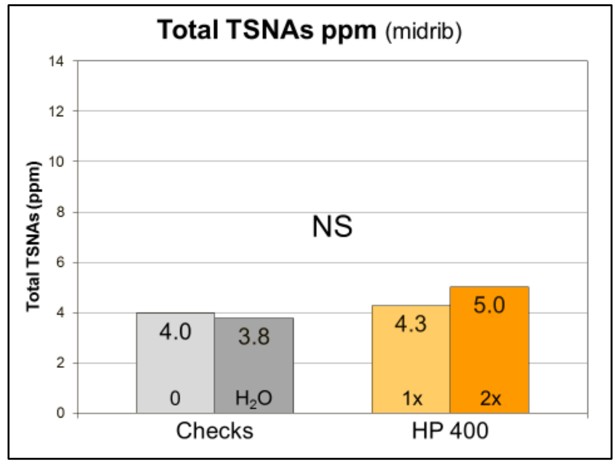


Figure 11: Effect of HP 400 sprays on NNK. **A.** Lamina NNK, 2015 **B.** Midrib NNK, 2015 **C.** Lamina NNK, 2016 **D.** Midrib NNK, 2016 **E.** Lamina NNK, years combined **F.** Midrib NNK, years combined

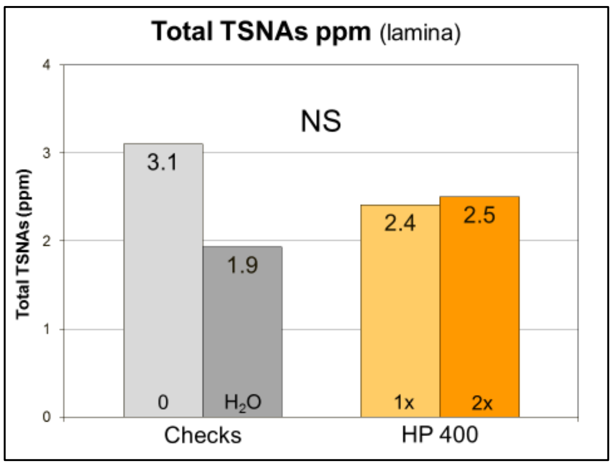
BDL = below detectable limit NS = not significant (p>0.05)



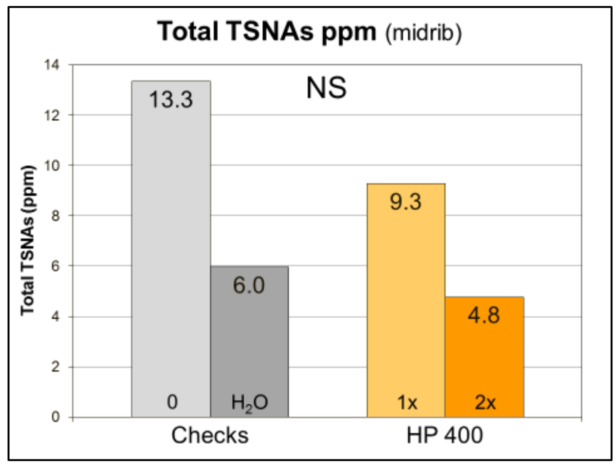
A. 2015



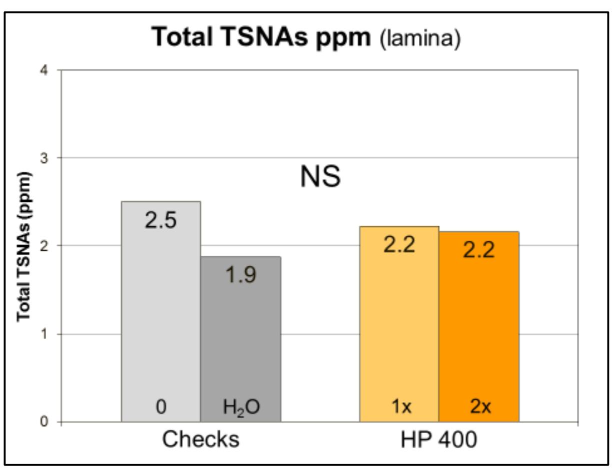
B. 2015



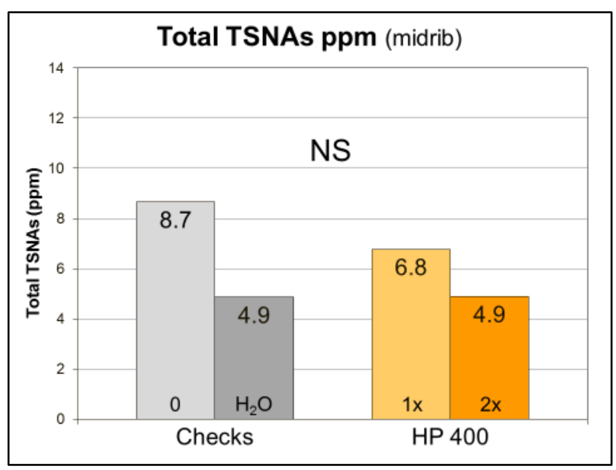
C. 2016



D. 2016



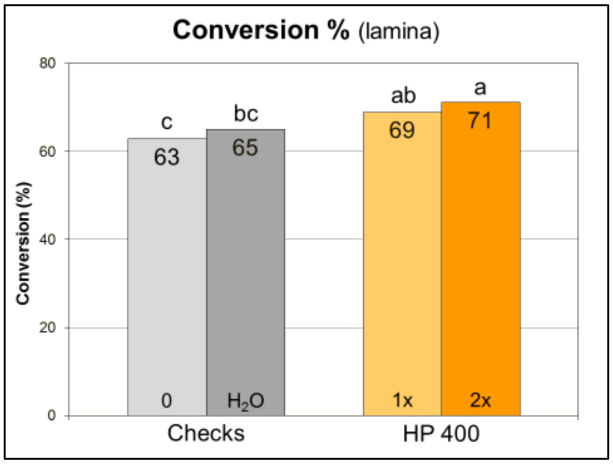
E. Years combined



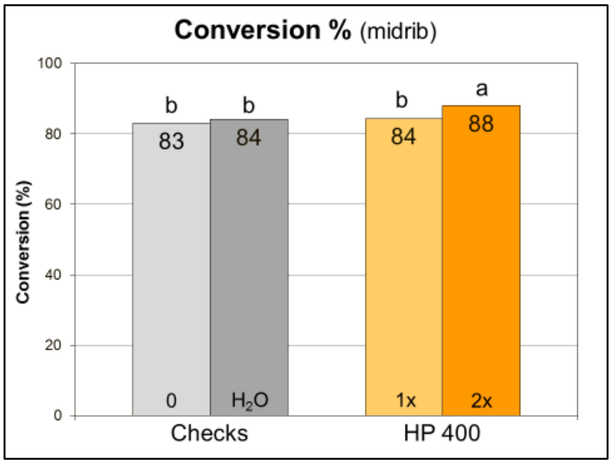
F. Years combined

Figure 12: Effect of HP 400 sprays on total TSNAS. **A.** Lamina total TSNAS, 2015 **B.** Midrib total TSNAS, 2015 **C.** Lamina total TSNAS, 2016 **D.** Midrib total TSNAS, 2016 **E.** Lamina total TSNAS, years combined **F.** Midrib total TSNAS, years combined

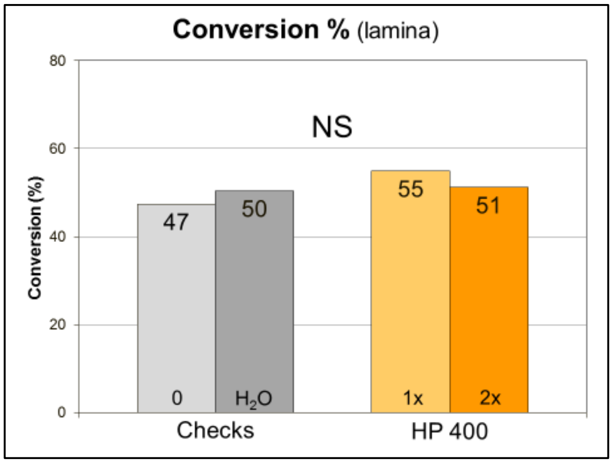
NS = not significant (p>0.05)



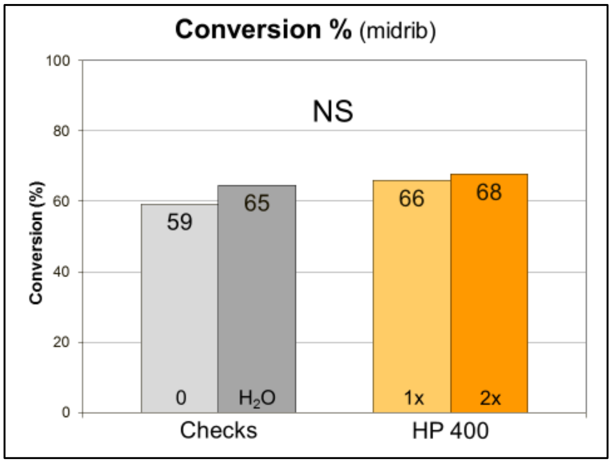
A. 2015



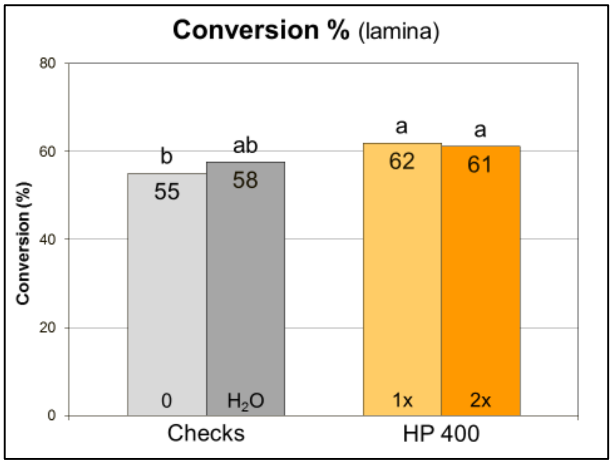
B. 2015



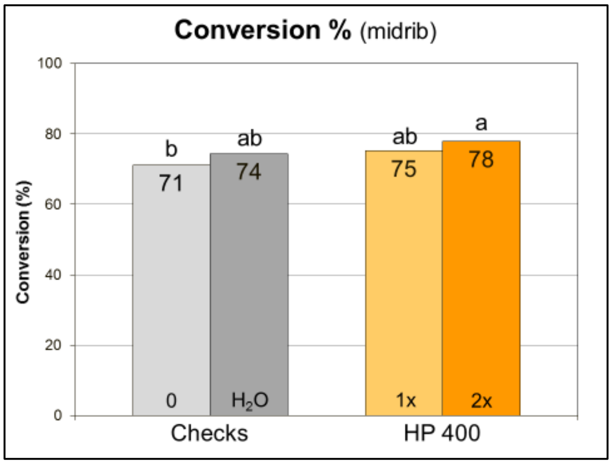
C. 2016



D. 2016



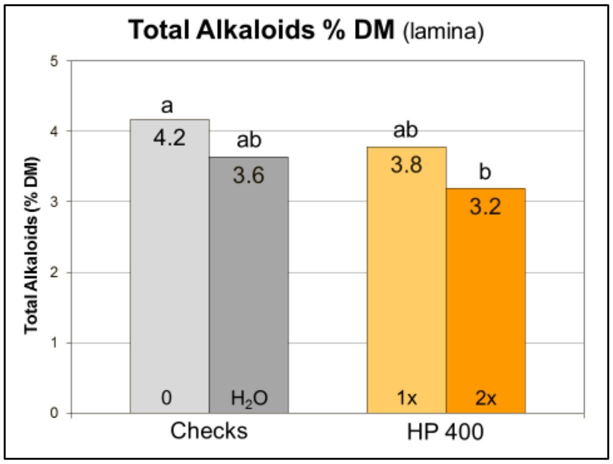
E. Years combined



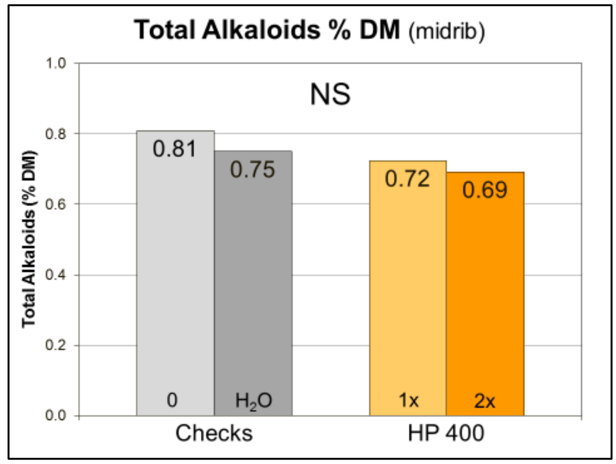
F. Years combined

Figure 13: Effect of HP 400 sprays on conversion. **A.** Lamina conversion, 2015 **B.** Midrib conversion, 2015 **C.** Lamina conversion, 2016 **D.** Midrib conversion, 2016 **E.** Lamina conversion, years combined **F.** Midrib conversion, years combined

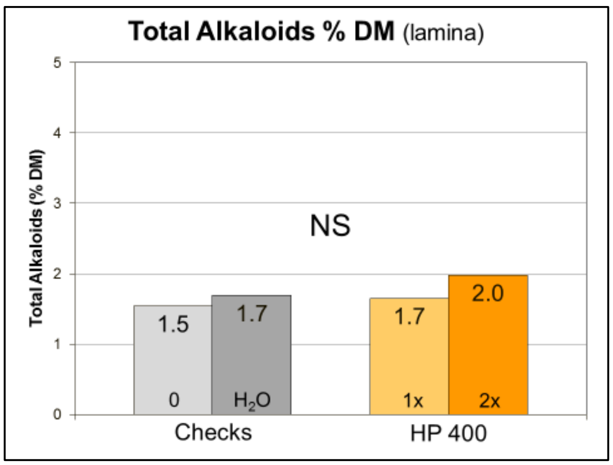
Bars with a common letter are not significantly different (p>0.05) NS = not significant (p>0.05)



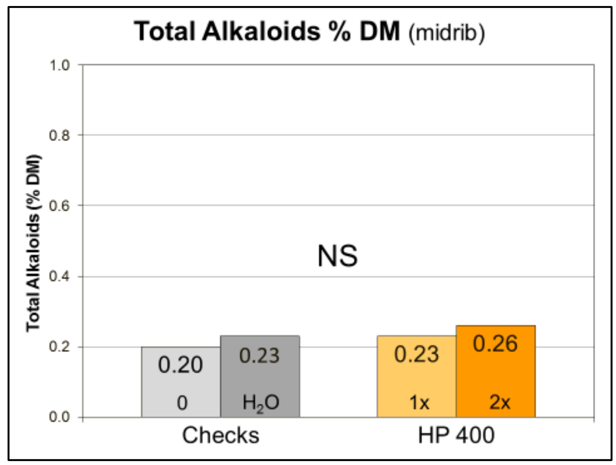
A. 2015



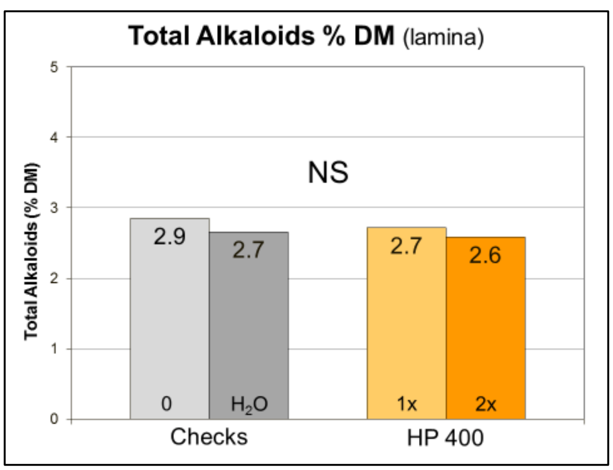
B. 2015



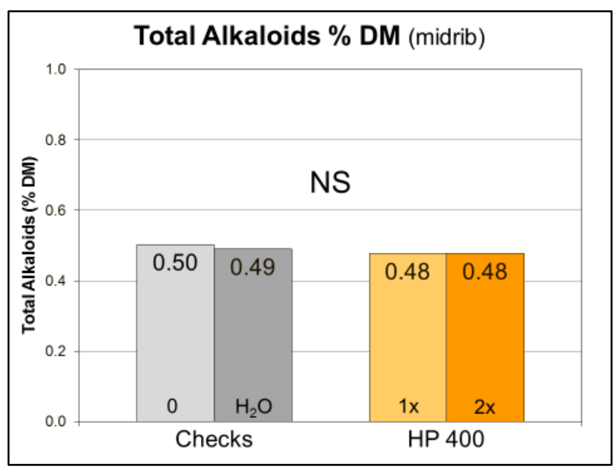
C. 2016



D. 2016



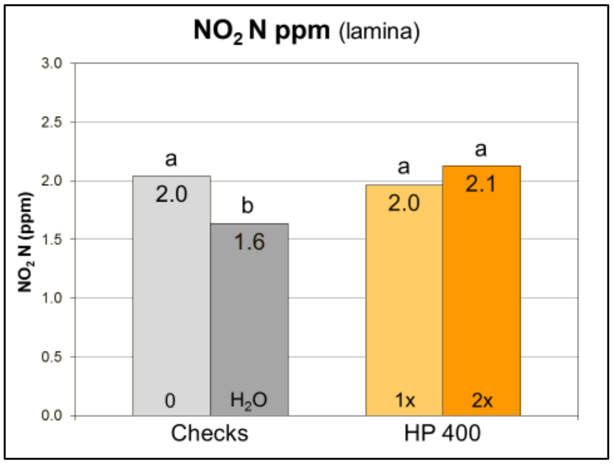
E. Years combined



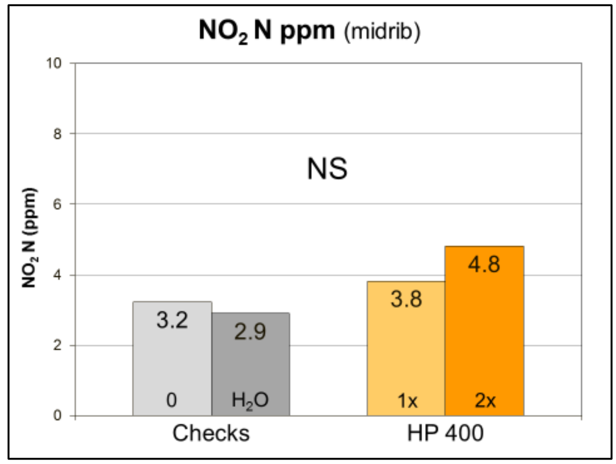
F. Years combined

Figure 14: Effect of HP 400 sprays on total alkaloids (TA). **A.** Lamina TA, 2015 **B.** Midrib TA, 2015 **C.** Lamina TA, 2016 **D.** Midrib TA, 2016 **E.** Lamina TA, years combined **F.** Midrib TA, years combined

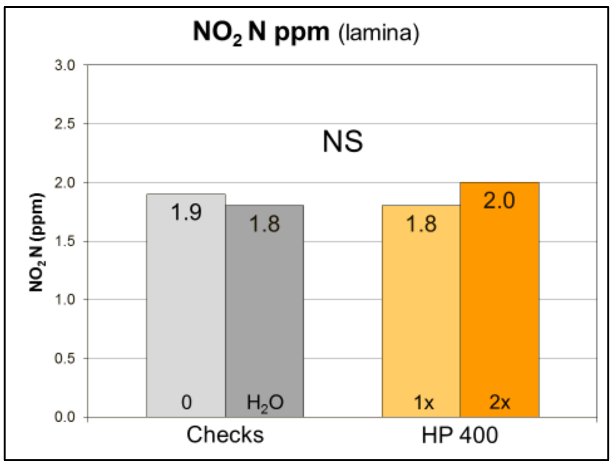
Bars with a common letter are not significantly different (p>0.05) NS = not significant (p>0.05)



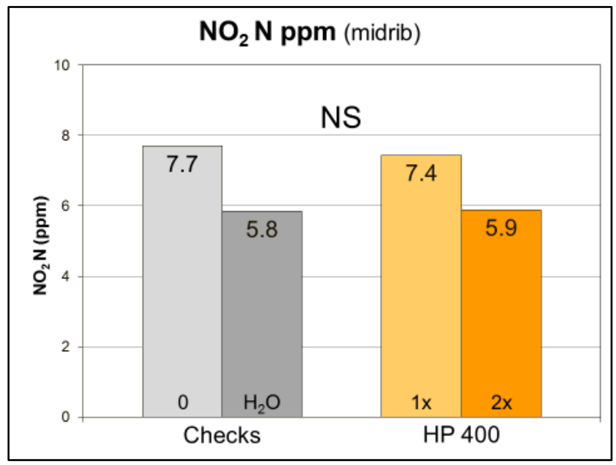
A. 2015



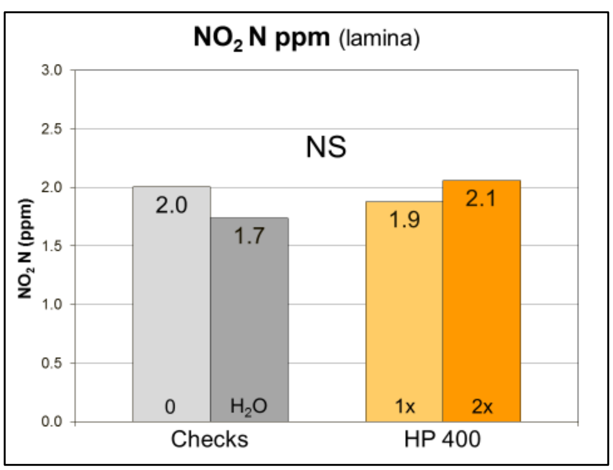
B. 2015



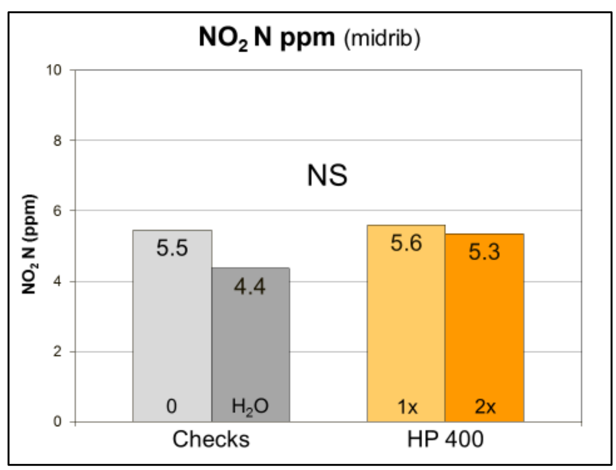
C. 2016



D. 2016



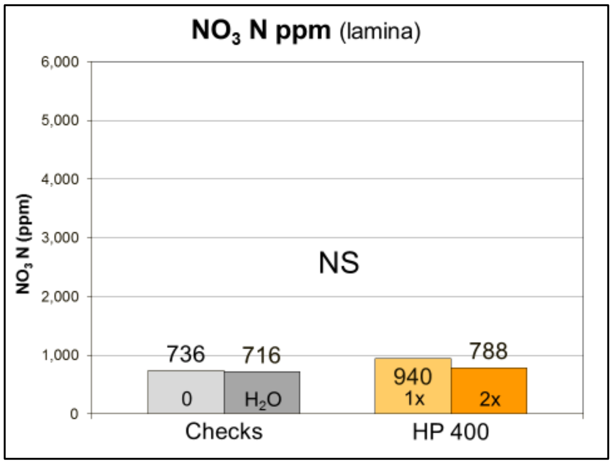
E. Years combined



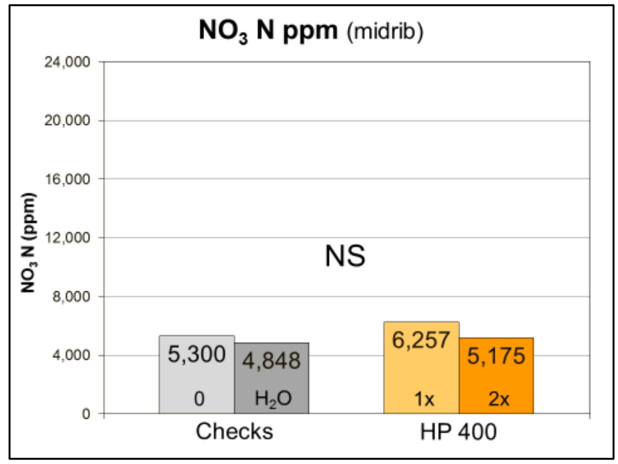
F. Years combined

Figure 15: Effect of HP 400 sprays on NO_2 N. **A.** Lamina NO_2 N, 2015 **B.** Midrib NO_2 N, 2015 **C.** Lamina NO_2 N, 2016 **D.** Midrib NO_2 N, 2016 **E.** Lamina NO_2 N, years combined **F.** Midrib NO_2 N, years combined

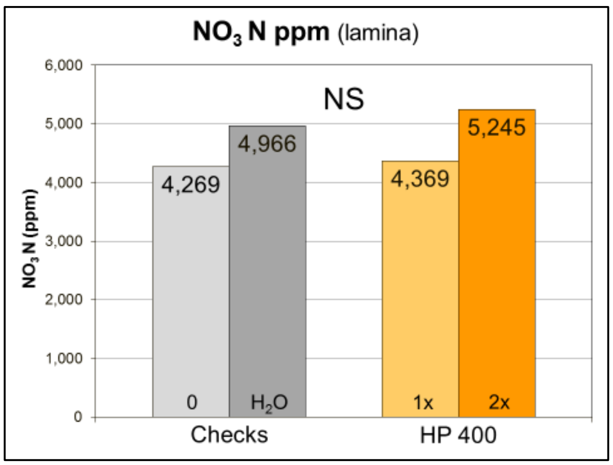
Bars with a common letter are not significantly different (p>0.05)   NS = not significant (p>0.05)



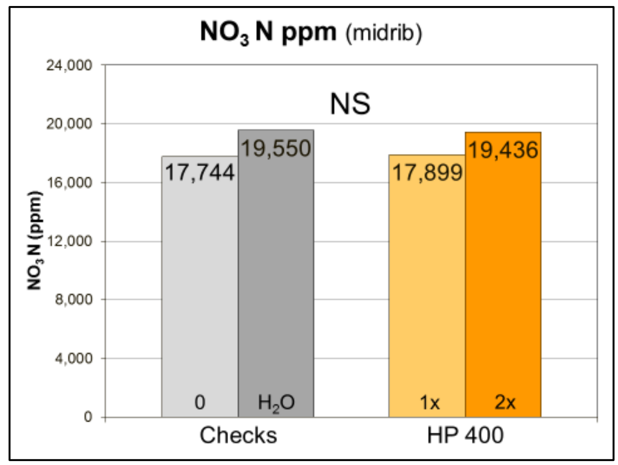
A. 2015



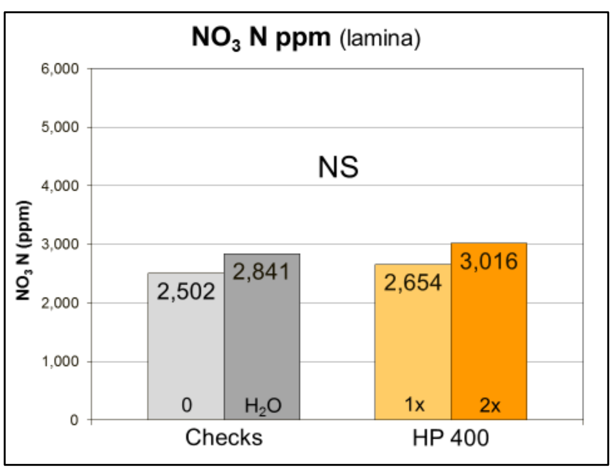
B. 2015



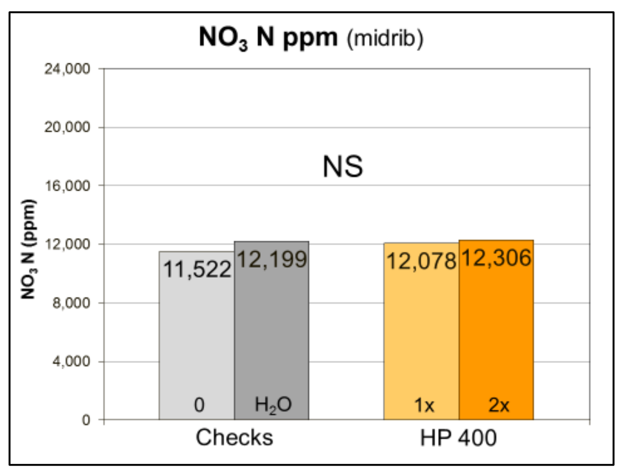
C. 2016



D. 2016



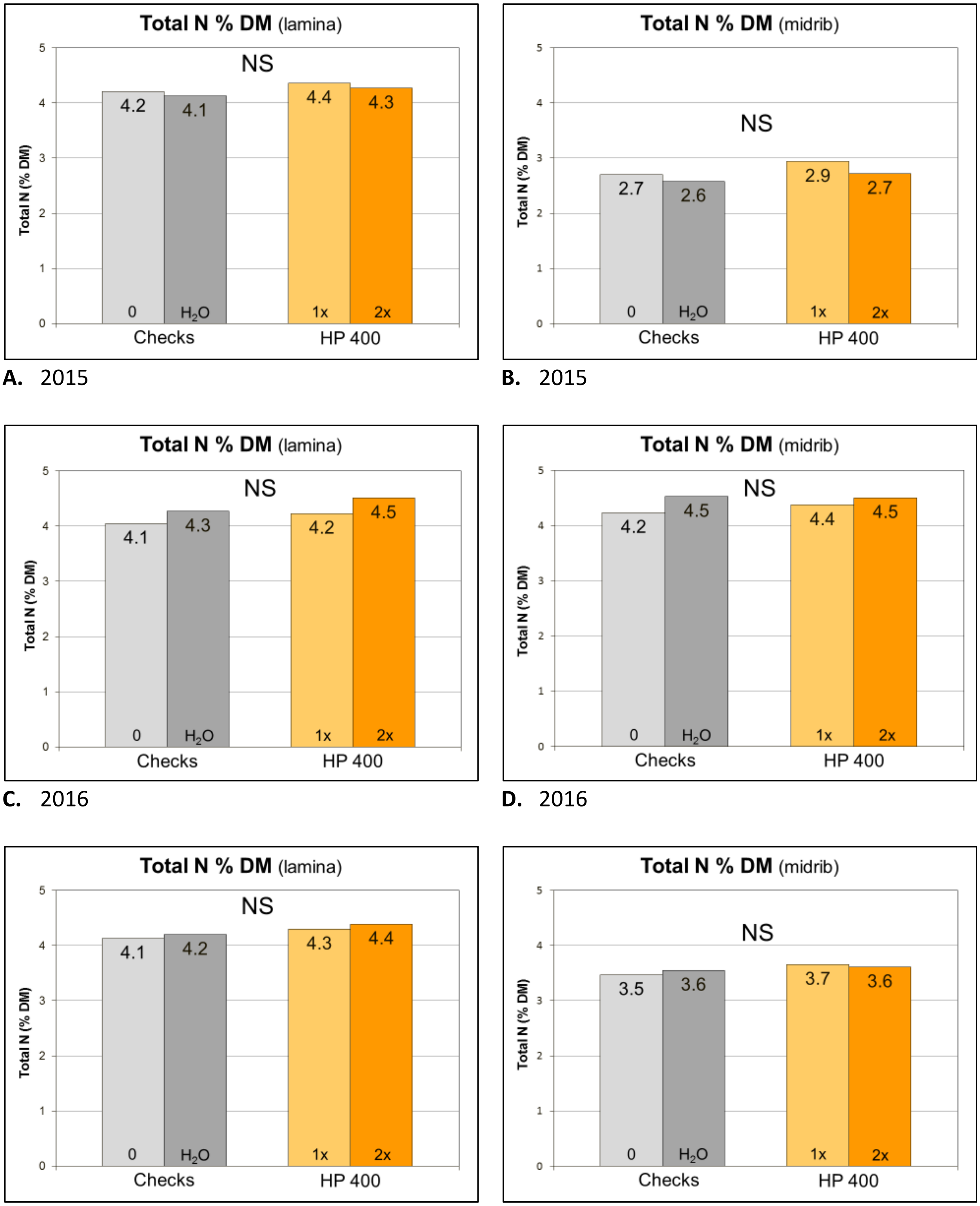
E. Years combined



F. Years combined

Figure 16: Effect of HP 400 sprays on NO_3 N. **A.** Lamina NO_3 N, 2015 **B.** Midrib NO_3 N, 2015 **C.** Lamina NO_3 N, 2016 **D.** Midrib NO_3 N, 2016 **E.** Lamina NO_3 N, years combined **F.** Midrib NO_3 N, years combined

NS = not significant ($p>0.05$)



A. 2015 **B.** 2015

C. 2016 **D.** 2016

E. Years combined **F.** Years combined

Figure 17: Effect of HP 400 sprays on total N. **A.** Lamina total N, 2015 **B.** Midrib total N, 2015 **C.** Lamina total N, 2016 **D.** Midrib total N, 2016 **E.** Lamina total N, years combined **F.** Midrib total N, years combined

NS = not significant (*p*>0.05)