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

Title: The Effects of Cytokinin Application on the Accumulation of Tobacco-Specific Nitrosamines (2015, 2016 seasons)

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Report type: Final report

Lay Summary: This study was designed to test whether the plant hormone cytokinin could be used to reduce TSNAs (Tobacco Specific Nitrosamines) in burley tobacco. Cytokinins affect the nitrogen metabolism and the antioxidant capacity of the plant, so in theory could affect TSNA accumulation. This report covers both the 2015 and 2016 seasons. A pilot study showed promising results, so these studies involved an increase in the cytokinin concentration to test whether this causes an even stronger suppression of TSNA formation without adversely affecting curing. Plants were sprayed the day before harvest, with a low and a high (5x low) rate. There were no significant differences between the cytokinin treatments and the controls, for any of the TSNAs except for midrib NNK (Nicotine-derived Nitrosamine Ketone, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) in 2016, where the low rate of cytokinin appeared to increase NNK, but this effect was not consistent and can probably be discounted. The only other difference was in the 2016 lamina total nitrogen, where both cytokinin rates increased total nitrogen relative to the unsprayed check, but not relative to the water check. 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.

Introduction

Rationale

The goal of this study was to test whether spraying burley tobacco with the synthetic cytokinin benzyladenine is an efficient, cost-effective method for lowering TSNAs. Cytokinins regulate many aspects of plant growth and development. For example, cytokinins increase nitrogen utilization and the antioxidative capacity of the plant, and can delay senescence. We hypothesize that short cytokinin treatments alter the nitrogen metabolism and cellular antioxidative capacity, possibly lowering TSNA accumulation, while not retarding chlorophyll loss significantly. 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.

KTRDC sponsored a two-year pilot study for this project, as a proof of concept: we were not certain that cytokinin application would have any effect at all on TSNA accumulation. The first year results were promising: we found that the cytokinin application indeed lowered TSNA levels. We also found that the cytokinin concentration used does not delay senescence but instead promotes the chlorosis of leaves, suggesting that it promotes the senescence process, a potentially beneficial effect for the curing of tobacco leaves. This suggested that higher cytokinin concentrations could be used without causing any senescence delays. Now that we had proof of concept, we planned tests to establish the most suitable application. In 2015 and 2016, we increased the cytokinin concentration to test whether this causes an even stronger suppression of TSNA formation without adversely affecting curing. If we can find an effective spray treatment, this will be the cheapest, simplest and most reliable way to reduce TSNAs.

The long term objective was to establish the most suitable cytokinin application to reduce TSNA accumulation. The short term objective was to test the effect of a higher cytokinin rate on TSNA accumulation.

Background

Cytokinins (CKs) are plant hormones that regulate cell division, elongation and differentiation, and are therefore essential for every aspect of plant growth and development (Mok and Mok 2001). For example, CKs control the development of meristems and vasculature, and play an important role in senescence and nutrient allocation (Mok and Mok 2001; Gan and Amasino 1995). The compounds defined as CKs include a large and diverse group of substances, most of which are adenine derivatives. Benzylaminopurine (BA) is a synthetic CK that affects plant growth and development consistent with the known functions of endogenous CKs.

TSNA accumulation is primarily impacted by the levels of precursors; secondary alkaloids and nitrite. A number of factors interact to determine the levels of these precursors, and the many aspects of nitrogen metabolism play a key role.

Three physiological effects caused by CKs are of particular interest for the proposed project:

1. CK-induced changes in nitrogen remobilization;
2. CK-induced changes in cellular antioxidative capacity; and
3. CK-induced inhibition of senescence (e.g. chlorophyll retention).

Whereas the first two effects of CKs may reduce TSNA formation, the third effect of CKs is undesirable and would have a negative impact on the quality of air-cured tobacco. CKs are known to control the levels of the first enzyme in nitrate assimilation, nitrate reductase (NR) (Yu *et al.* 1998). The activities of NR and nitrite reductase (NiR), the second enzyme of the nitrate assimilation pathway, are often co-regulated (Faure *et al.* 1991), but the CK effect on NiR levels in tobacco has not been described. The next key enzyme of the nitrate assimilation pathway is glutamine synthase (GS), and its levels are regulated by CKs at least in *Arabidopsis thaliana* (J. Kurepa and J. Smalle, unpublished). Although a number of factors influence TSNA accumulation, one of the major factors is the amount of nitrite accumulated during air-curing (Burton *et al.* 1994). CK treatments prior to curing may increase the flow through the nitrogen assimilation pathway, thus reducing the nitrite level and potentially reducing the accumulation of TSNAs.

Increasing the antioxidant capacity of tobacco prior to curing is another possible approach to reducing TSNA accumulation (Rundlöf *et al.* 2000). CKs are known to induce the activity of some of the antioxidative enzymes in different plant species, and are also known to have antioxidative chemical properties on their own (Wilson-Garcia *et al.* 2008; Zavaleta-Mancera *et al.* 2007; Rattan 2004).

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.

Treatments

Previous results suggested that spraying post-harvest, in addition to the pre-harvest field spray, did not significantly increase the cytokinin effect on TSNA accumulation. We therefore planned to use only the pre-harvest spray.

The treatments were two controls (water control and unsprayed) and two rates of an aqueous solution of BA; the rate used previously and a higher (5x) rate. The water control and both rates of BA were applied at the rate of 50 gallons/acre, 27 ml/ plant, 24 hours before harvest in with a backpack sprayer 2015, and with a high clearance tractor in 2016. Because the BA is a growth regular, the rates used are extremely low; 0.0008 and 0.004 oz/acre.

1. No spray (unsprayed control)
2. Water spray (solvent control), 50 gallons/acre, 24 hours before harvest
3. 2013 rate – 0.2 µM BA in 50 gallons/acre water, 24 hours before harvest
   0.45 mg/L, 0.000016 oz/gallon, 0.0008 oz/acre of product
4. 5x 2013 rate – 1 µM BA in 50 gallons/acre water, 24 hours before harvest
   2.25 mg/L, 0.000079 oz/gallon, 0.004 oz/acre of product

Design

The design was a factorial with four randomized complete blocks, each 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. Six days before transplanting, we applied 200 lb/acre N as urea, and 350 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 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 1); 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 cytokinin sprays and water control were applied with a backpack sprayer the day before harvest, August 26th (see *Treatments* for details). The study was harvested 31 days after topping, on August 27th (Figure 3). 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 (Figure 4), when it was picked up and put onto a rail wagon (Figure 5) which was parked in the barn until housing four days after harvest (August 31st).

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 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. 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.

As in 2015, we had an unusual spectrum of pests and diseases. There was a heavy infestation of Japanese beetles (Figure 1); 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 cytokinin sprays and the water control were applied one day before harvest, August 31st (see *Treatments* for details). We used a high clearance tractor (Figure 2), unlike 2015, when we applied the

treatments with a backpack sprayer. The study was harvested 31 days after topping, on September 1st (Figure 3). 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 (Figure 4), when it was picked up and put onto a rail wagon which was parked in the barn until housing five days after harvest, on September 6th (Figure 5).

Sampling for molecular analysis

Samples for molecular analysis were taken from the railwagon the day after harvest (Figure 6). We took two subsamples from each plot; the two center sticks (2 and 3) of the five sticks. The two center plants on these sticks (plants 3 and 4 of six plants) were sampled by taking two leaf discs with a 12.5 mm / ½ inch diameter cork borer (Figure 7), giving us eight replicates of four leaf discs each. We sampled the third leaf from the top of the plant; two discs on either side of the midrib, one finger length from the tip, midway between the leaf margin and midrib (Figure 8).

Samples were placed on ice while a plot was being sampled, then placed in an aluminum foil folded packet and dropped into liquid nitrogen. They were stored in a -80°C freezer awaiting processing.

Sampling and sample preparation for chemical analysis

The tobacco was taken down in January both years and sampled 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.

Procedure – Molecular Laboratory

No molecular analyses were done, because cytokinin application did not consistently impact any of the constituents measured (see *Results* for details).

Procedure – Analytical Laboratory

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, mostly below the detectable limit)

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.

Procedure – Statistical Analysis

PROC MIXED of SAS 9.1 (SAS Institute, Cary, NC, USA) was used for an analysis of variance appropriate for a factorial 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 transformations were done where necessary (Table 1), prior to means separation procedures. Means were separated according to protected Fisher's least significant difference.

Results and Discussion

TN 90H seed

We have ascertained that the TN 90H seed used in 2016 was contaminated with another variety. Data from a two-year study (2015, 2016) incorporating both TN 90LC and TN 90H, are presented in Figures 9A – 9D. 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 9A). Conversion for TN 90LC in both years was consistent with that expected for a low converter variety. 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. 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; 35% instead of the usual 70-80%. 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 9E, 9F). All plants grown from the 2008 seedlot had >90% conversion and (nicotine + nornicotine) about 1% DM (green oval, Figure 9E), as would be expected for TN 90H. However, the 2014 seedlot was clearly a mixture of TN 90H, with >90% conversion and (nicotine + nornicotine) around 1% DM (green oval, Figure 9F), and a low alkaloid, low converter line with mostly <10% conversion and (nicotine + nornicotine) all around 0.1% DM (red oval, Figure 9F). 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 total TSNAs in 2105 (Figure 13A) 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 17A, 17B). 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 1.6-2.0 ppm in 2015 and 1.9-3.4 ppm in 2016 (Figure 13). If the correct TN 90H seed had been used, TSNAs would certainly have been even higher.

There were no significant differences between cytokinin treatments and checks for NNN (*N*-Nitrosonornicotine), NAT (*N*-Nitrosoanatabine) and total TSNAs (Figures 10, 11, 13). The only significant difference was in the 2016 midrib NNK (Nicotine-derived Nitrosamine Ketone, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone), where the low rate of cytokinin appeared to increase NNK, but this effect was not consistent and can probably be discounted (Figure 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.

Lamina conversion ranged from 63-67% in 2015 and 47-55% in 2016. There were no significant differences between cytokinin treatments and checks (Figure 14).

Total alkaloids (TAs) ranged from 3.8-4.2% DM in 2015 and 1.5-1.9% DM in 2016. There were no significant differences between cytokinin treatments and checks (Figure 15). For lamina TAs in 2015, there was a significant difference between the checks, but neither check was significantly different from the cytokinin treatments. The water check was significantly lower than the unsprayed check. While this difference between the checks 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 a water spray one day 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. 3.3 ppm. There were no significant differences between cytokinin treatments and checks (Figure 16).

Nitrate nitrogen was unusually low in 2015, but was at normal levels in 2016 (646-849 ppm lamina 2015, 4,725-5,627 ppm lamina 2016). There were no significant differences between cytokinin treatments and checks (Figure 17).

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: 2.4-2.8% DM vs. 4.2-4.6% DM (Figure 18). The only significant difference between cytokinin treatments and checks was in the 2016 lamina, where both cytokinin treatments increased total N relative to the unsprayed check, but not relative to the water check (Figure 18C).

One might speculate that in a season more conducive to TSNA accumulation than 2015, or that if the correct high converter had been grown in the more favorable 2016 season, cytokinin application might have had a significant impact on reducing TSNAs. However, the results so far have been disappointing.

Conclusions

Despite initial promising results, in two years we have not been able to show a reduction in TSNAs as a result of cytokinin sprays. However, we have had one season very unfavorable for TSNA accumulation, and one season where the variety grown had alkaloid and conversion levels too low for appreciable TSNA accumulation. It is possible that in a more typical season, with the correct variety, cytokinins may be efficacious.

Plans for Future Work

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

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Figures and Tables

Table 1: Effect of cytokinin 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	0.538	NS	log	0.461	NS	log	0.306	NS
NNN	Midrib	log	0.717	NS	log	0.393	NS	log	0.308	NS
NAT	Lamina	log	0.365	NS	log	0.371	NS	log	0.170	NS
NAT	Midrib	log	0.901	NS	log	0.329	NS	log	0.289	NS
NNK	Lamina	log	0.295	NS	log	0.357	NS	log	0.835	NS
NNK	Midrib	log	BDL [a]	NS	log	0.0376	*	log	0.059	NS
Total TSNAs	Lamina	log	0.527	NS	log	0.453	NS	log	0.294	NS
Total TSNAs	Midrib	log	0.737	NS	log	0.374	NS	log	0.294	NS
Conversion	Lamina	none	0.473	NS	none	0.325	NS	none	0.0996	NS
Conversion	Midrib	none	0.103	NS	none	0.589	NS	none	0.269	NS
Total Alkaloids	Lamina	none	0.0450	*	none	0.662	NS	none	0.478	NS
Total Alkaloids	Midrib	none	0.188	NS	none	0.468	NS	none	0.545	NS
NO_2 N	Lamina	log	0.296	NS	log	0.931	NS	log	0.833	NS
NO_2 N	Midrib	none	0.814	NS	none	0.200	NS	none	0.157	NS
NO_3 N	Lamina	none	0.533	NS	none	0.145	NS	none	0.0977	NS
NO_3 N	Midrib	none	0.565	NS	none	0.390	NS	none	0.466	NS
Total N	Lamina	none	0.739	NS	none	0.0156	*	none	0.123	NS
Total N	Midrib	none	0.604	NS	none	0.126	NS	none	0.519	NS

[a] = below detectable limit
NS = not significant ($p>0.05$) * = significant ($p>0.05$)

Figure 1: Japanese beetles

Figure 2: Spray application with a high clearance tractor

Figure 3: Harvesting

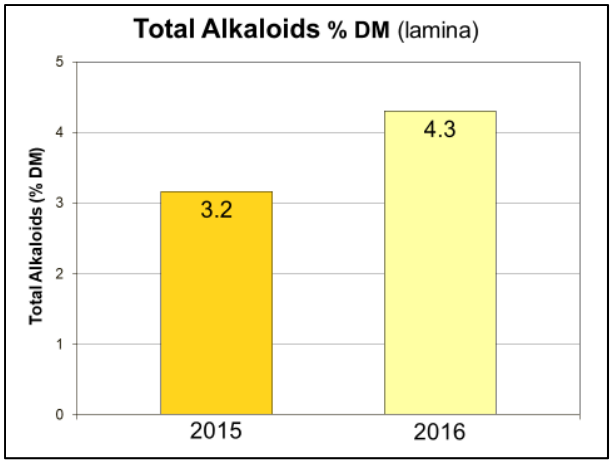
Figure 4: Tobacco sticked out after harvest

Figure 5: Railwagon in barn

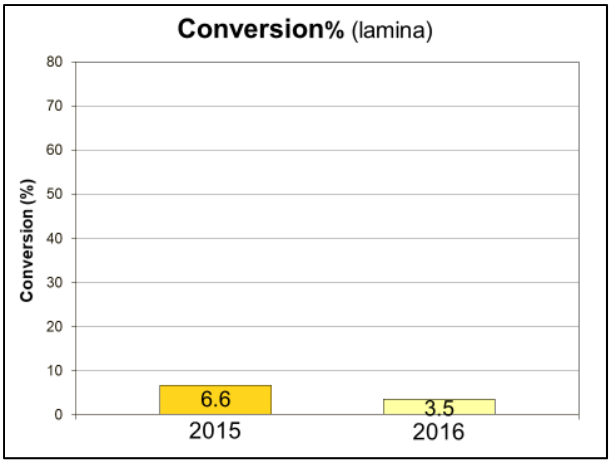
Figure 6: Plants to be sampled

Figure 7: Leaf disc samples

Figure 8: Sampling pattern



A. TN 90LC



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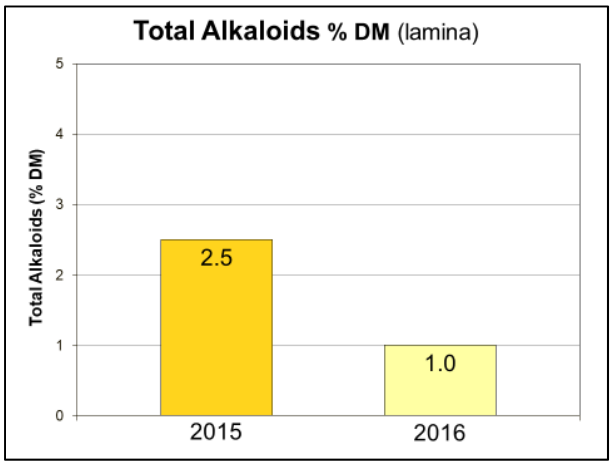
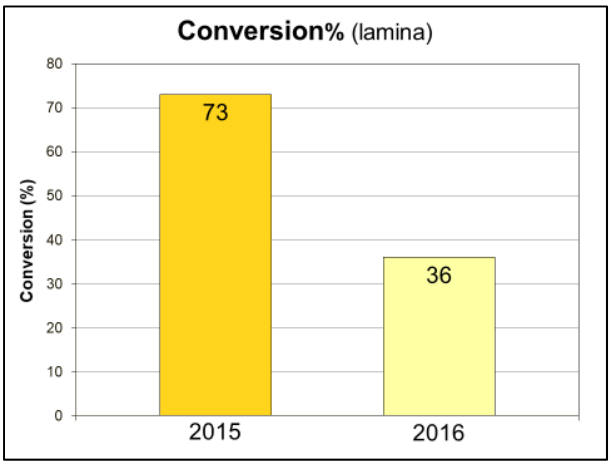
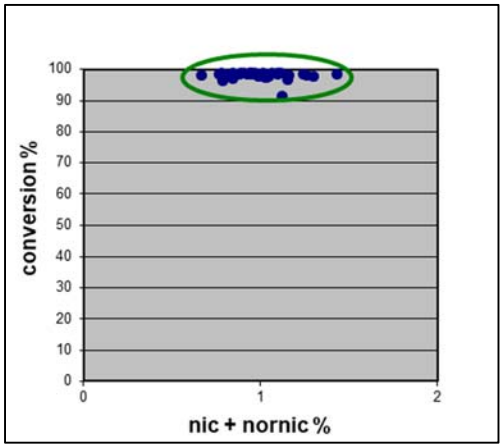


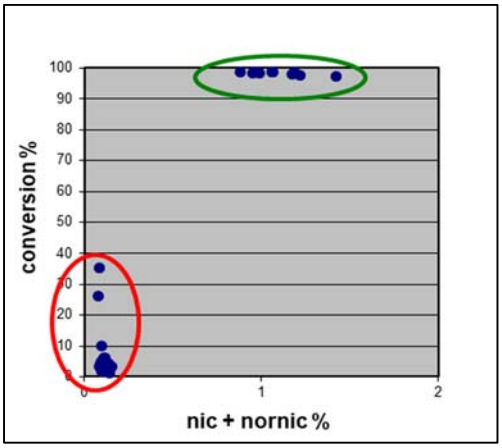
C. TN 90H



D. TN 90H

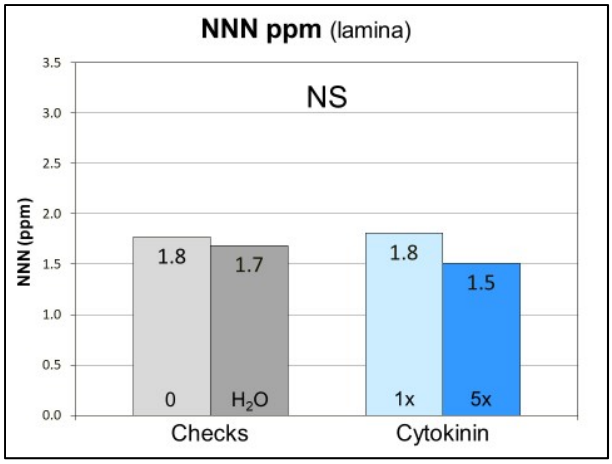


E. TN 90H seedlot grown in 2015 (2008 seed)

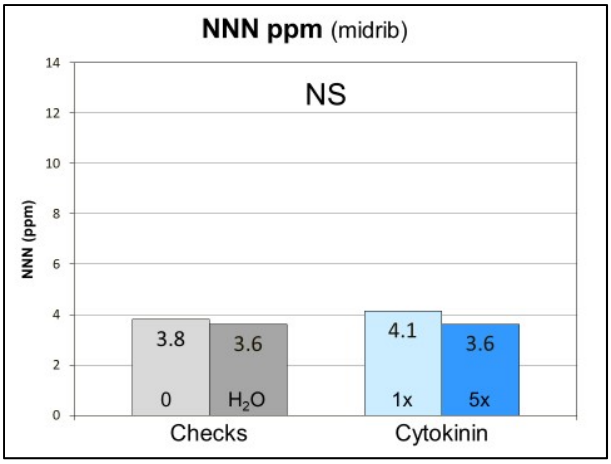


F. TN 90H seedlot grown in 2016 (2014 seed)

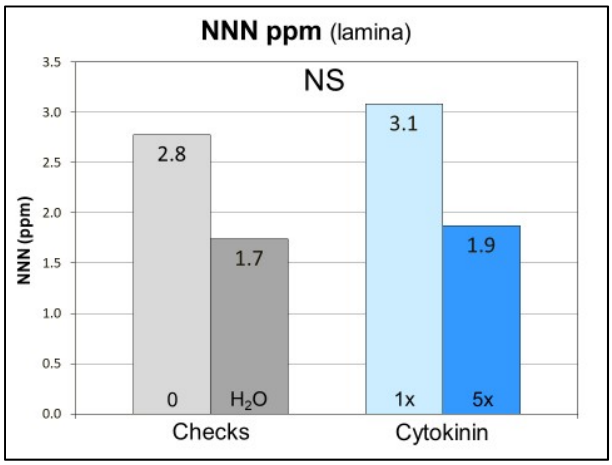
Figure 9: Total alkaloids and conversion in TN 90H and TN 90LC, 2015 and 2016, showing the difference between TN 90H seedlots (data from a different study). **A.** TN 90LC, lamina total alkaloids (TA) **B.** TN 90LC, lamina conversion **C.** TN 90H, lamina total alkaloids (TA) **D.** TN 90H, lamina conversion **E, F.** Scatter diagrams for individual TN 90H plants, two seedlots



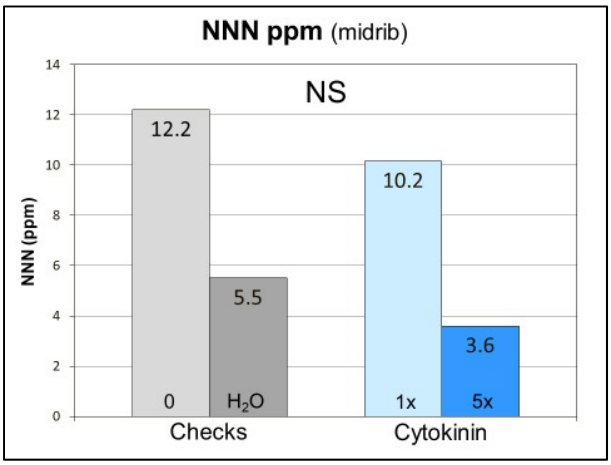
A. 2015



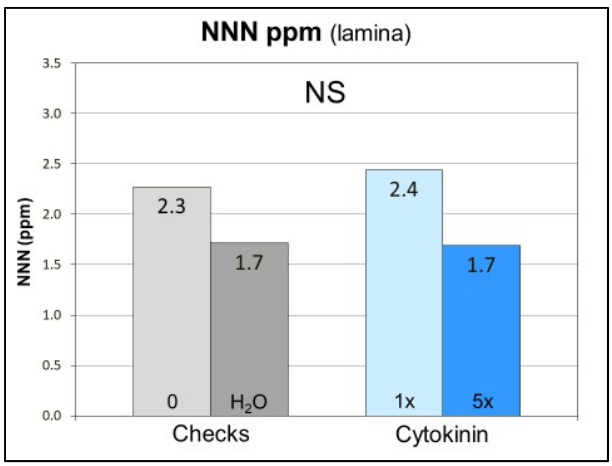
B. 2015



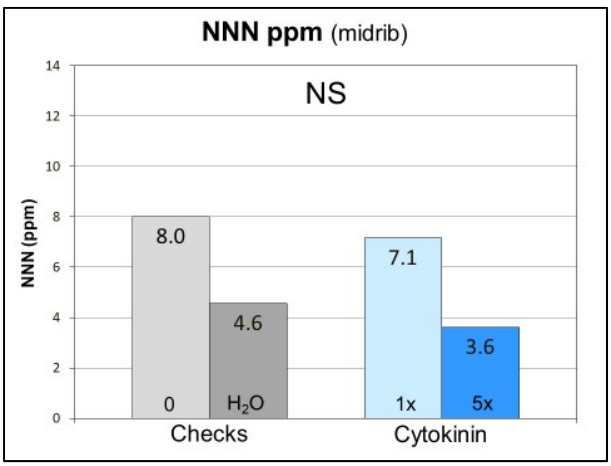
C. 2016



D. 2016



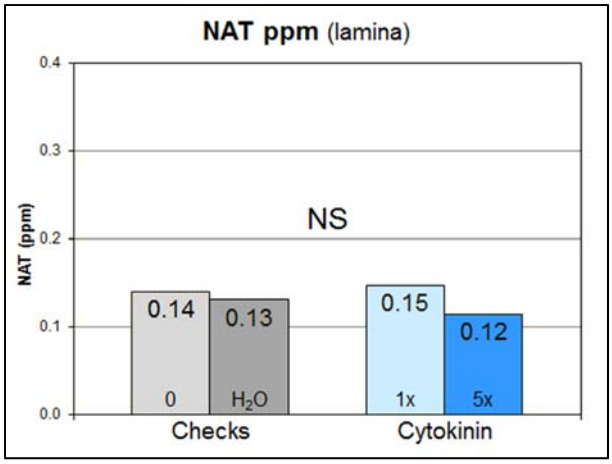
E. Years combined



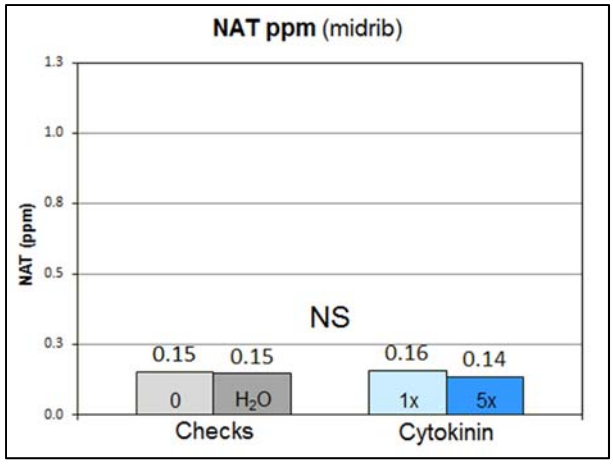
F. Years combined

Figure 10: Effect of cytokinin 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

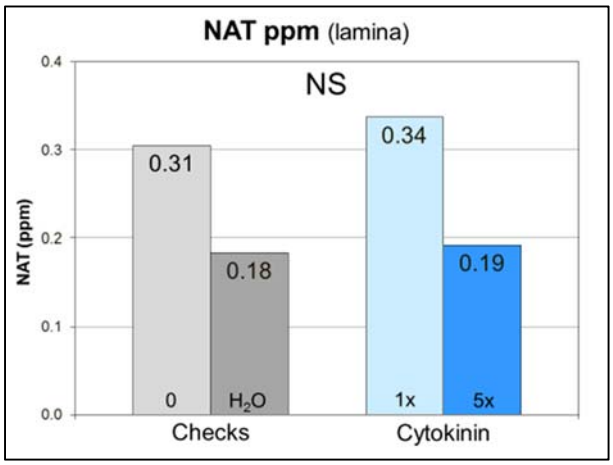
NS = not significant (p>0.05)



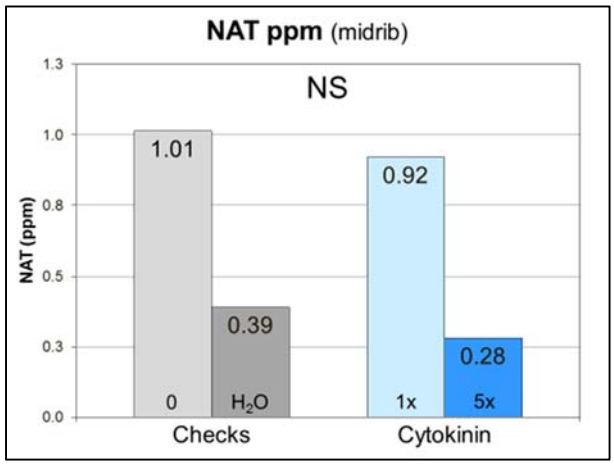
A. 2015



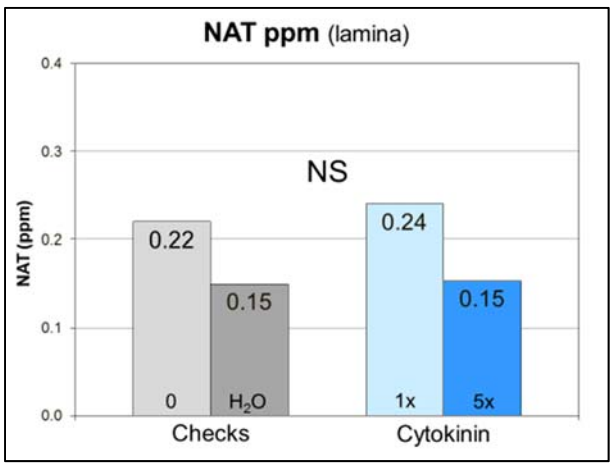
B. 2015



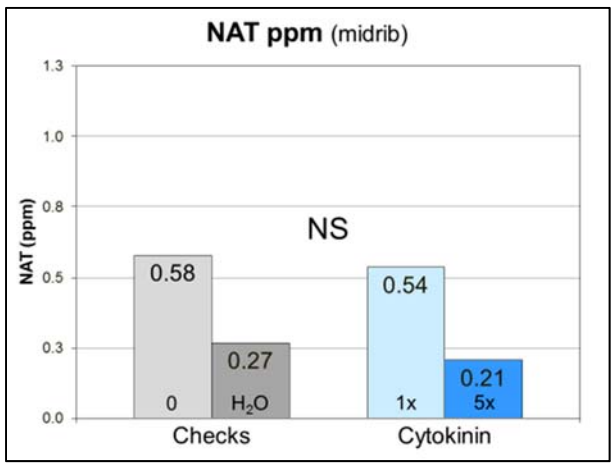
C. 2016



D. 2016



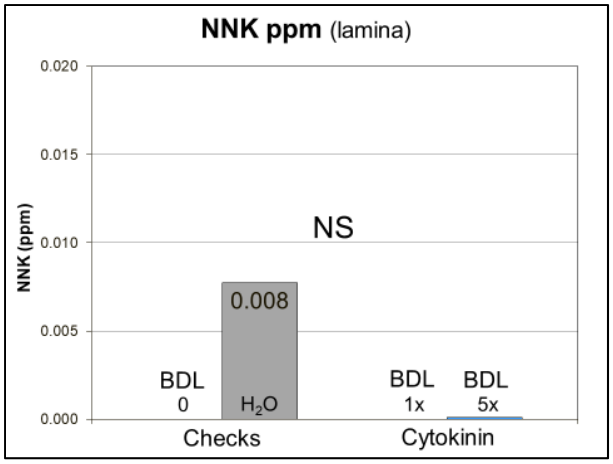
E. Years combined



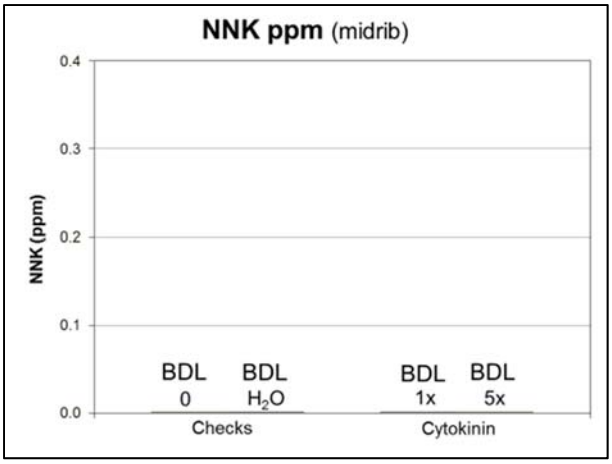
F. Years combined

Figure 11: Effect of cytokinin 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

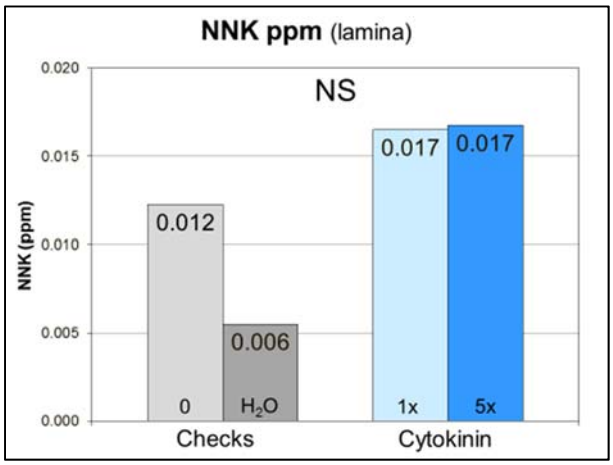
NS = not significant (p>0.05)



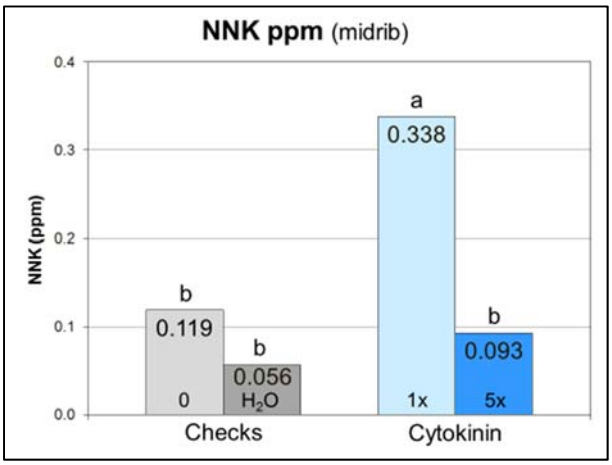
A. 2015



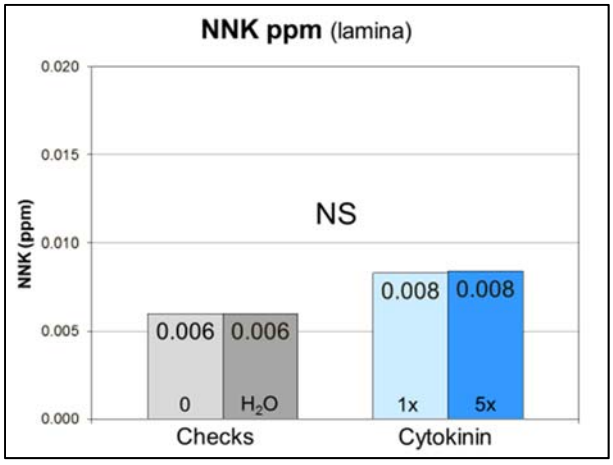
B. 2015



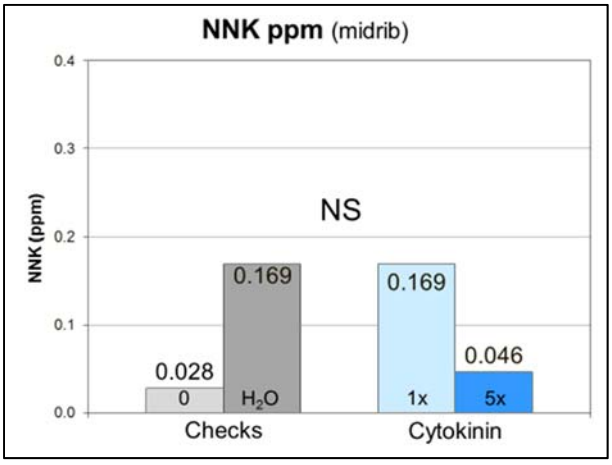
C. 2016



D. 2016



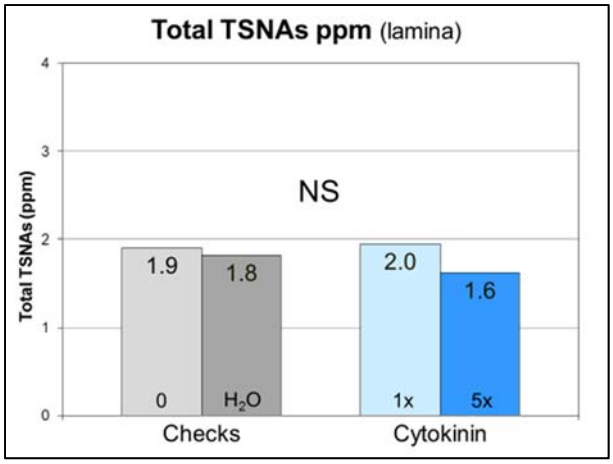
E. Years combined



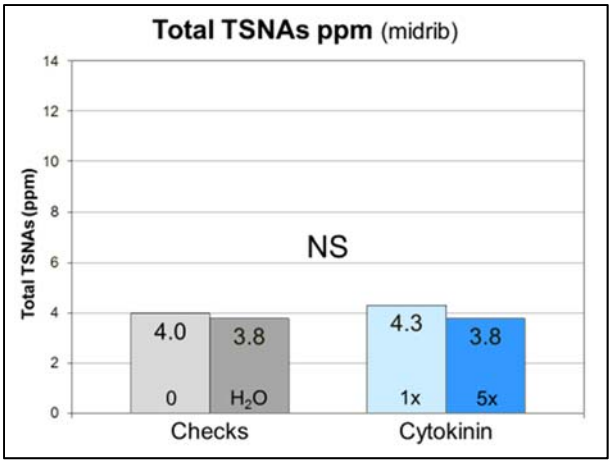
F. Years combined

Figure 12: Effect of cytokinin 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

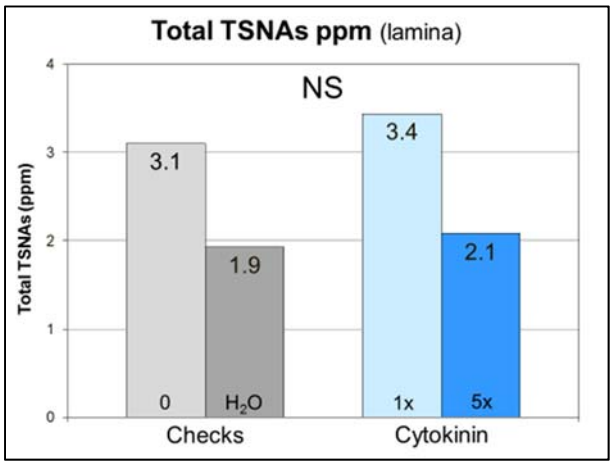
Bars with a common letter are not significantly different (p>0.05) NS = not significant (p>0.05) BDL = below detectable limit



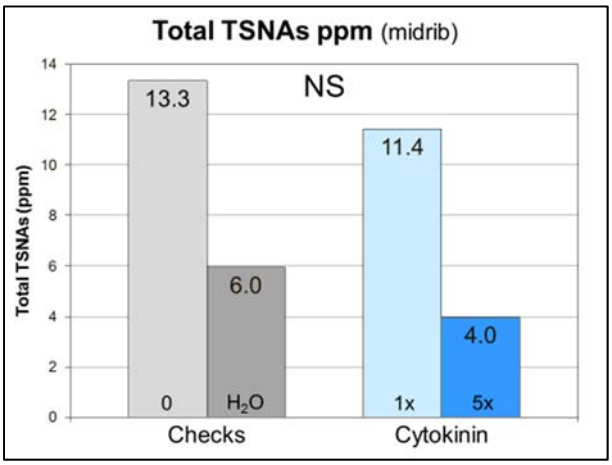
A. 2015



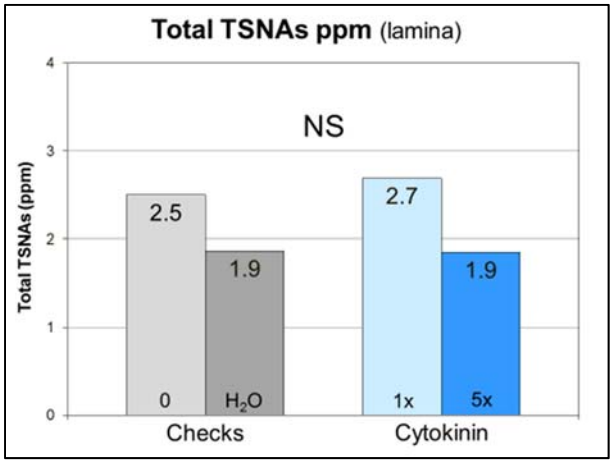
B. 2015



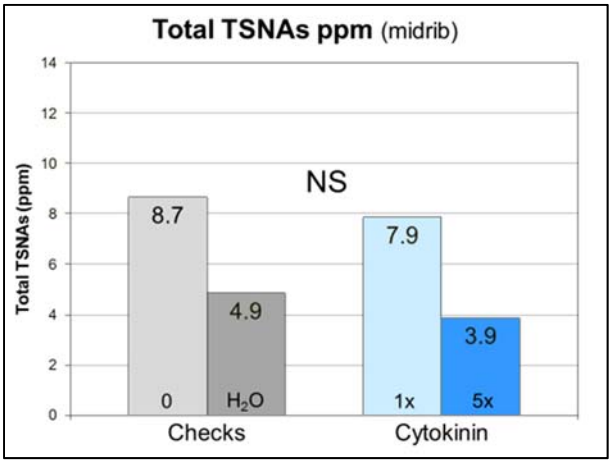
C. 2016



D. 2016



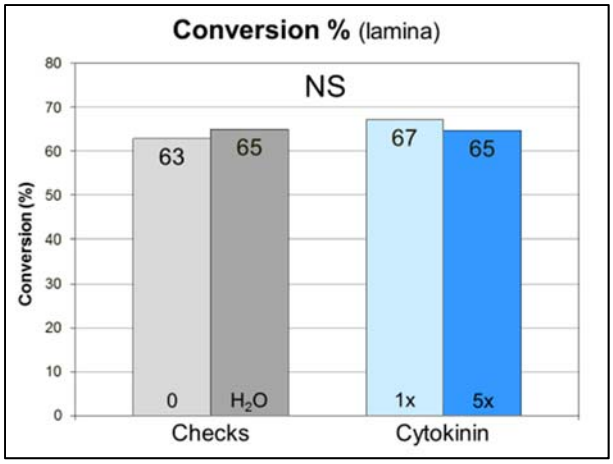
E. Years combined



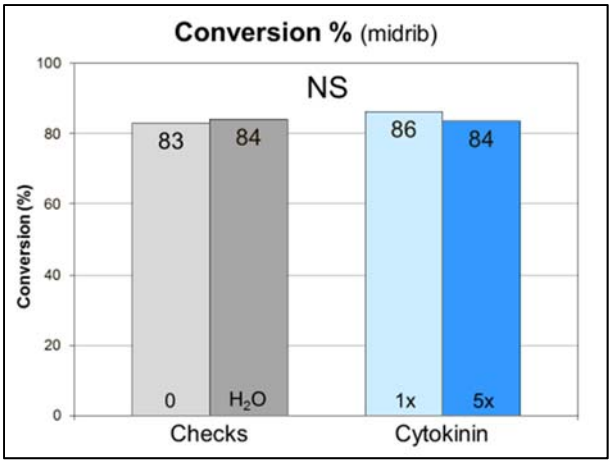
F. Years combined

Figure 13: Effect of cytokinin 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

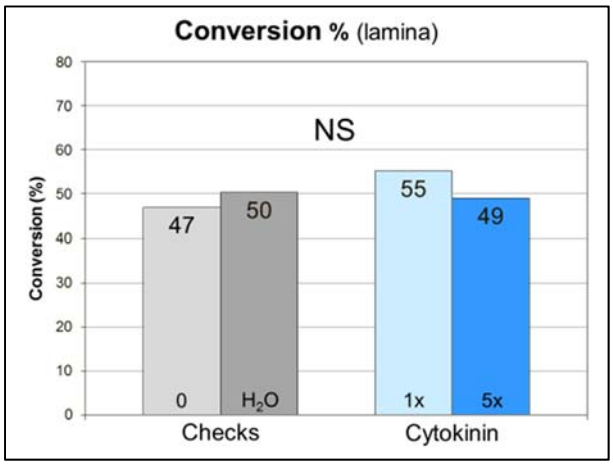
NS = not significant (p>0.05)



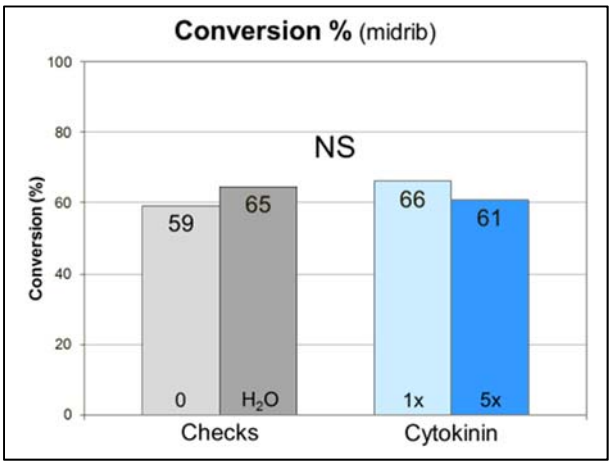
A. 2015



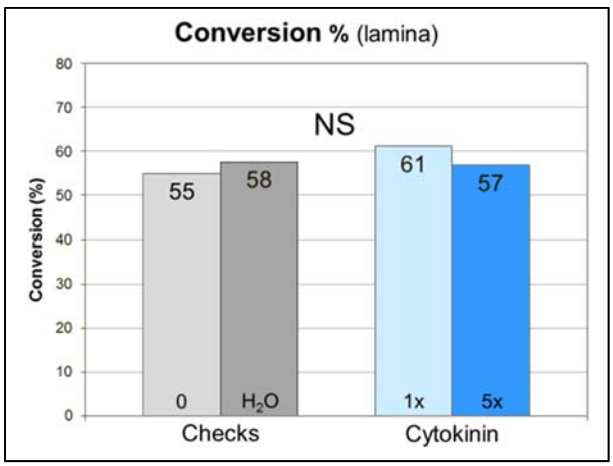
B. 2015



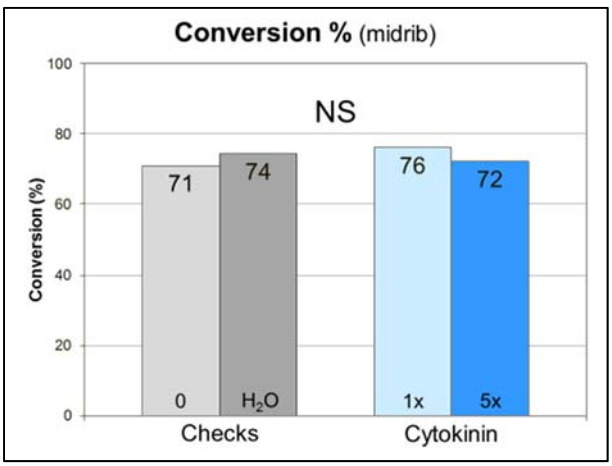
C. 2016



D. 2016



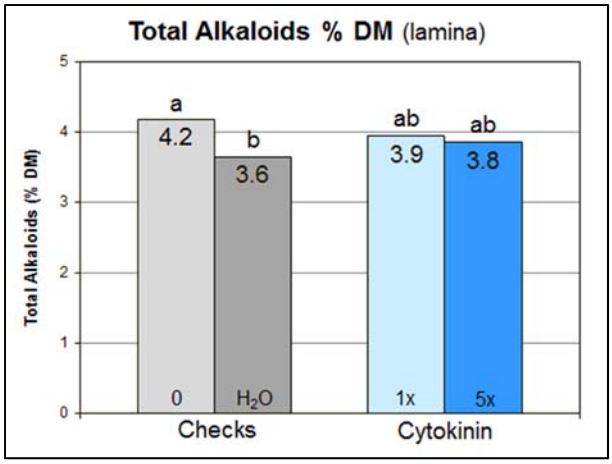
E. Years combined



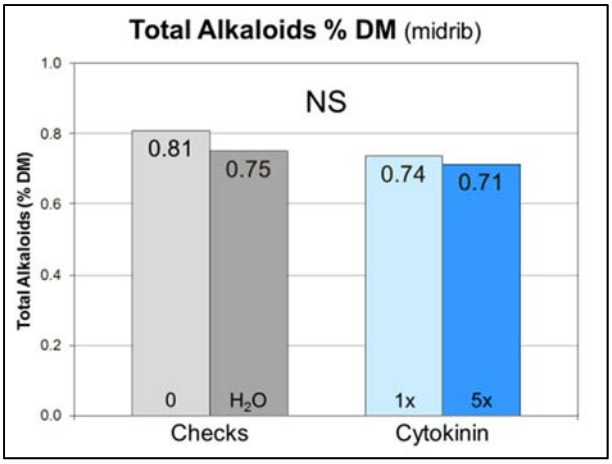
F. Years combined

Figure 14: Effect of cytokinin 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

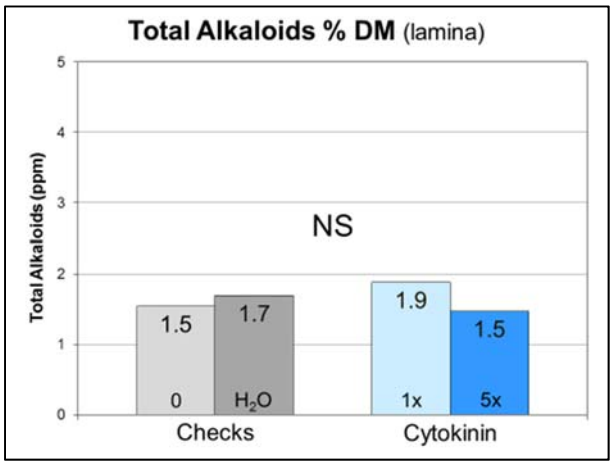
NS = not significant (p>0.05)



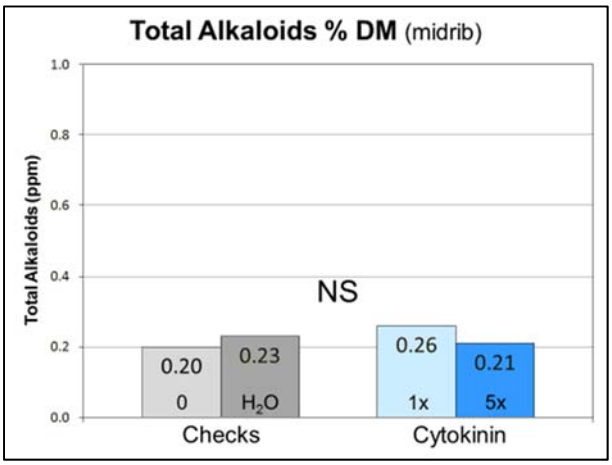
A. 2015



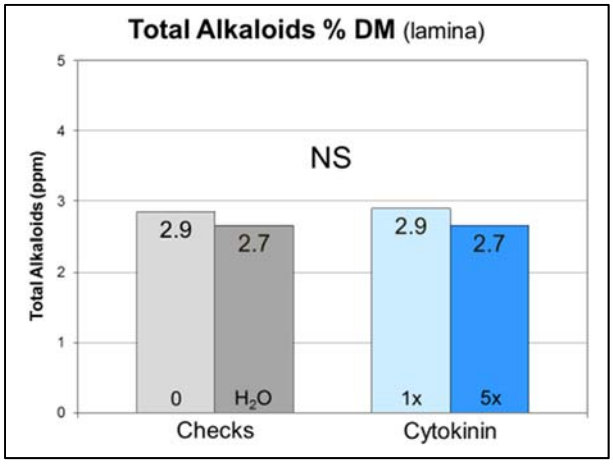
B. 2015



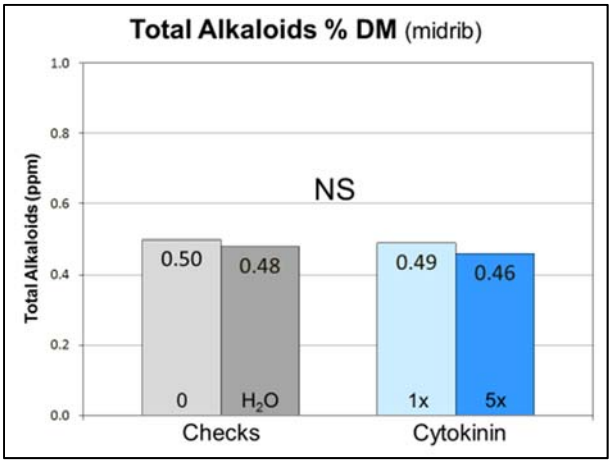
C. 2016



D. 2016



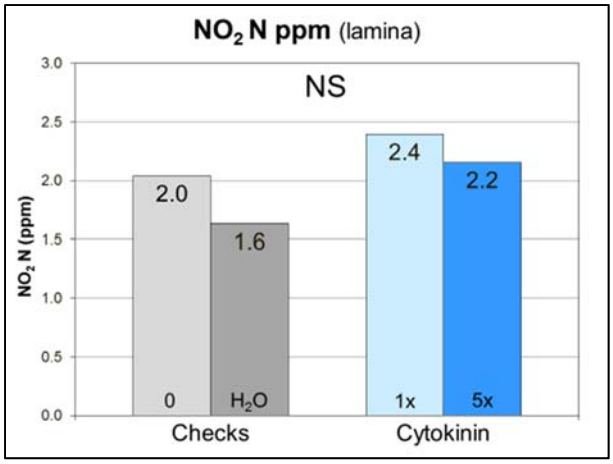
E. Years combined



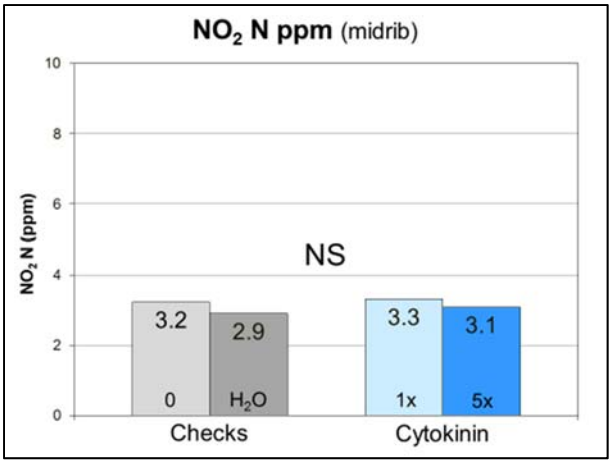
F. Years combined

Figure 15: Effect of cytokinin 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

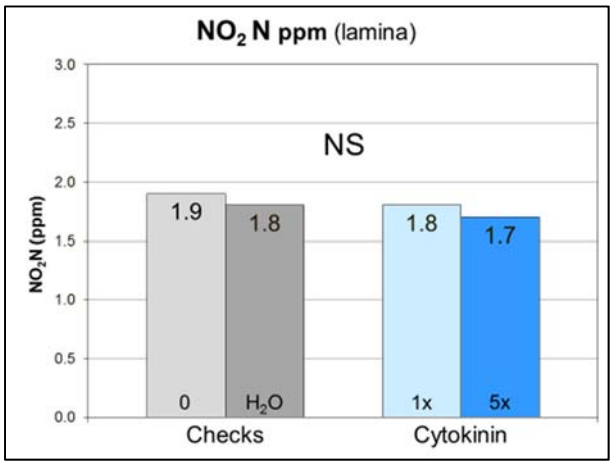
Bars with a common letter are not significantly different (p>0.05) NS = not significant (p>0.05)



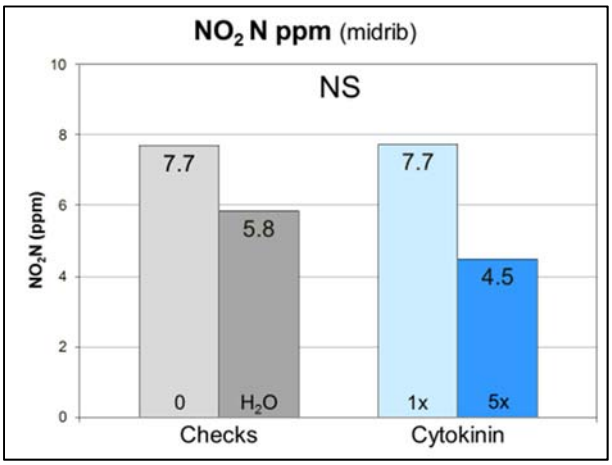
A. 2015



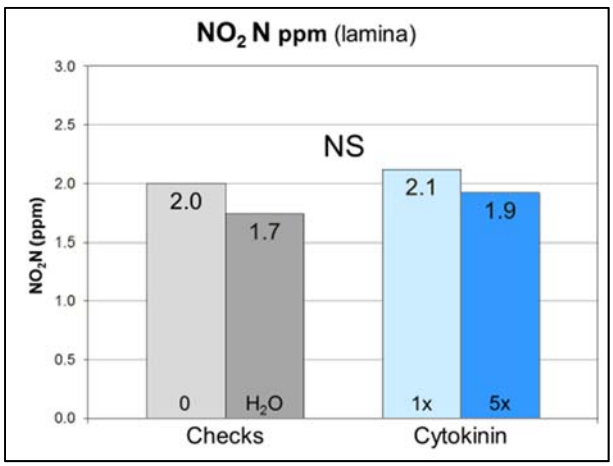
B. 2015



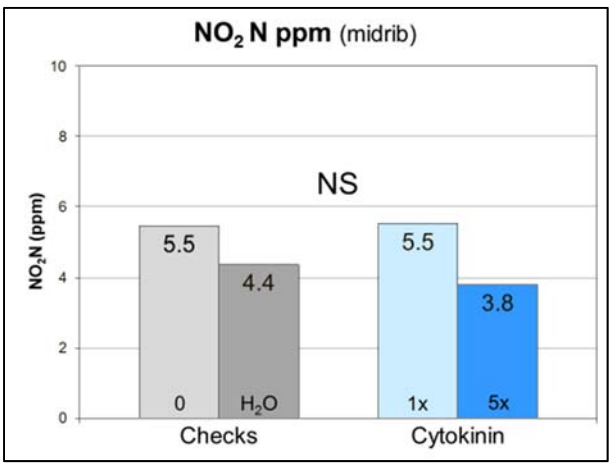
C. 2016



D. 2016



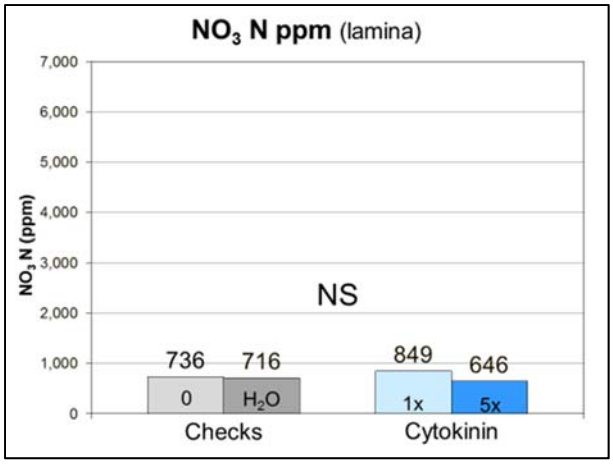
E. Years combined



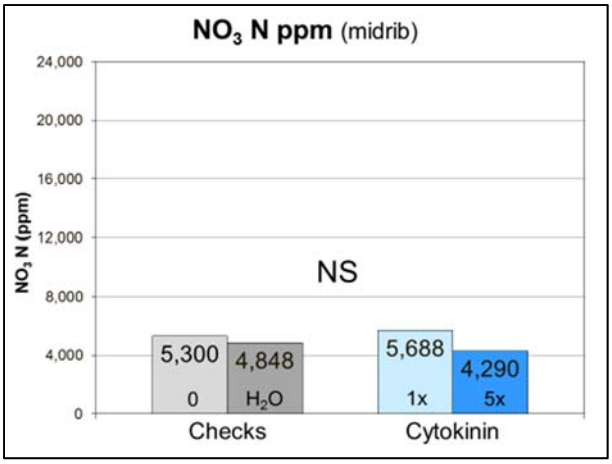
F. Years combined

Figure 16: Effect of cytokinin 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

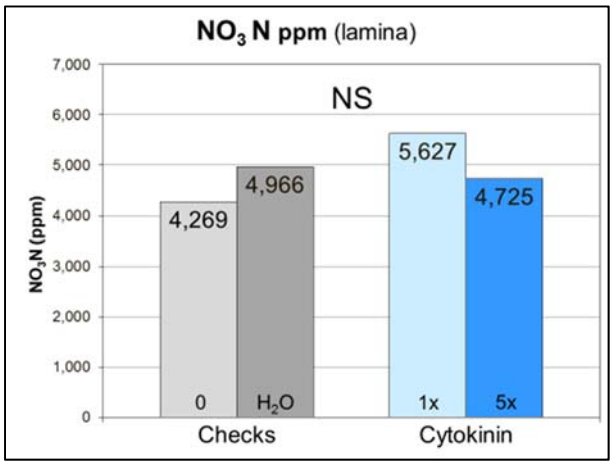
NS = not significant (p>0.05)



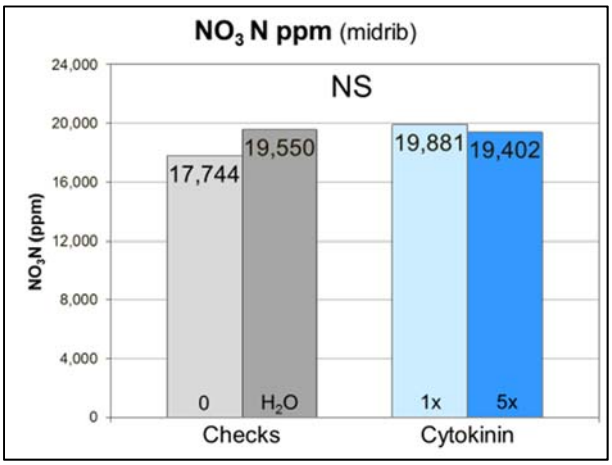
A. 2015



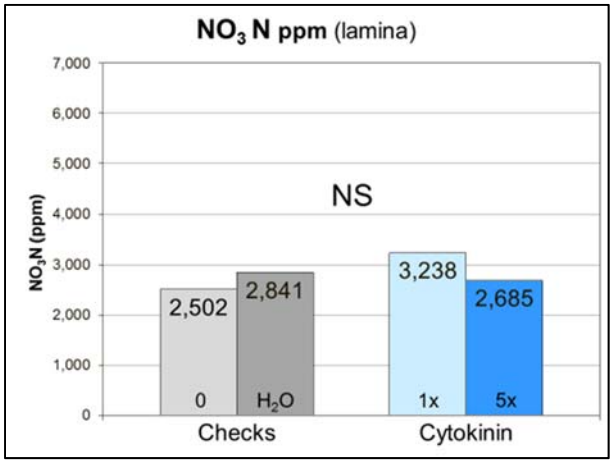
B. 2015



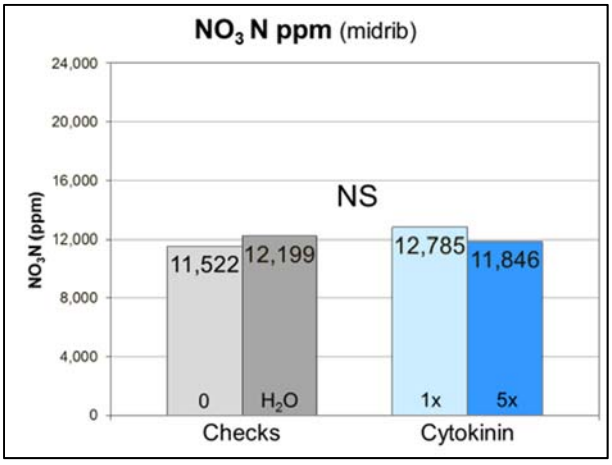
C. 2016



D. 2016



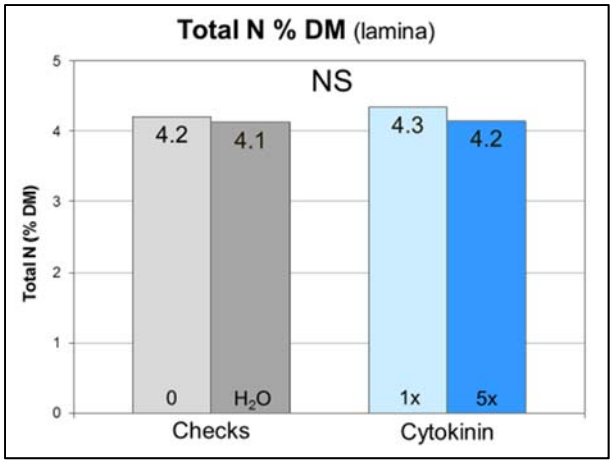
E. Years combined



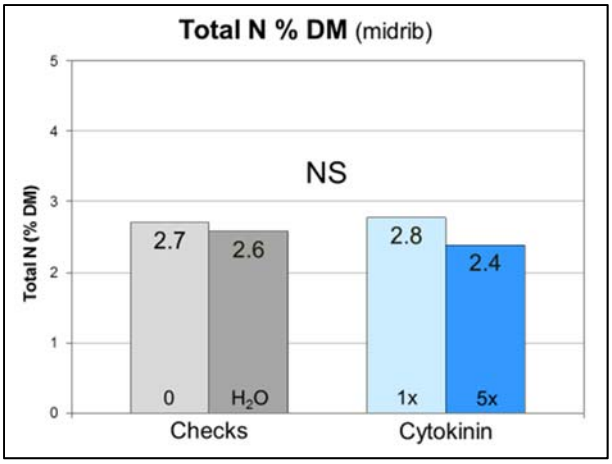
F. Years combined

Figure 17: Effect of cytokinin 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

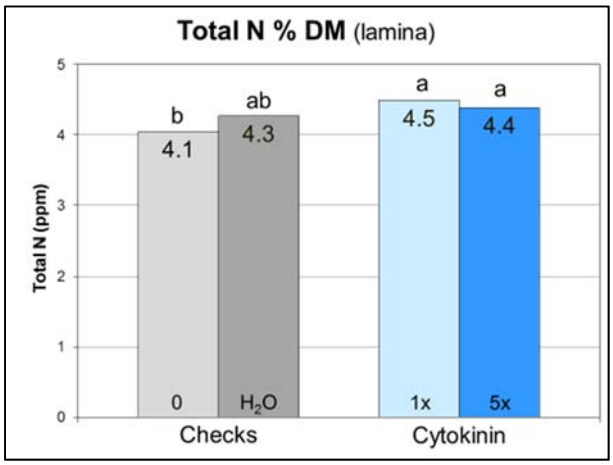
NS = not significant (p>0.05)



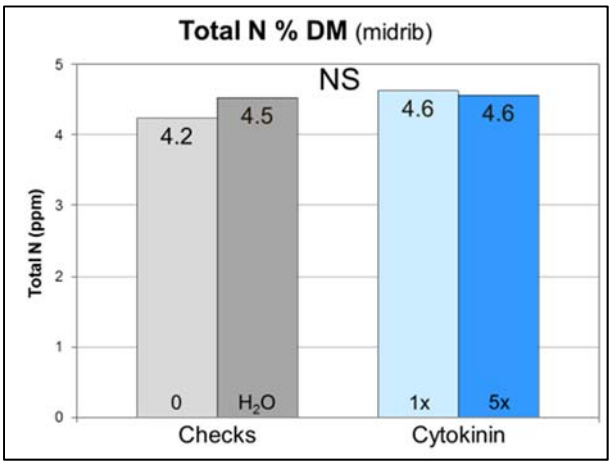
A. 2015



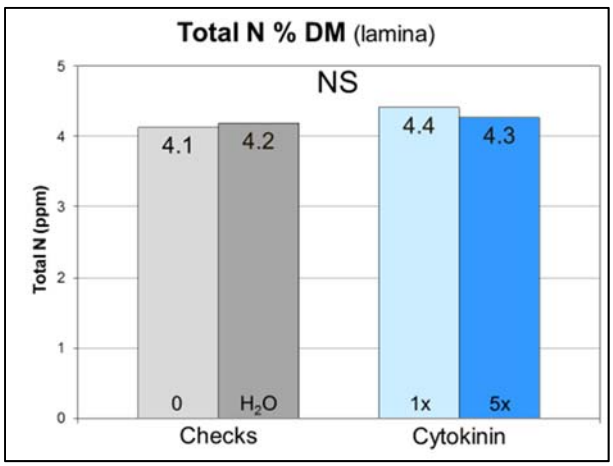
B. 2015



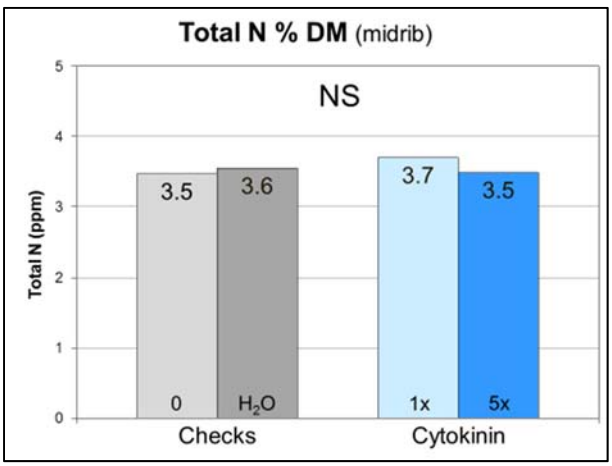
C. 2016



D. 2016



E. Years combined



F. Years combined

Figure 18: Effect of cytokinin 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

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