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

Title: **The Effects of Cytokinin Application on the Accumulation of Tobacco-Specific Nitrosamines (2015 season)**

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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. A pilot study showed promising results, so this year's study 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 variables. The only significant difference between any of the treatments was for lamina nitrate nitrogen, where the high rate of cytokinin reduced midrib nitrate nitrogen relative to the low rate; neither rate was significantly different from the controls. 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, cytokinin treatments may have an effect.

Introduction

Rationale

The goal of this study is 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 suggests that higher cytokinin concentrations could be used without causing any senescence delays. Now that we have proof of concept, we are planning tests to establish the most suitable application. This year, we propose to increase 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 is to establish the most suitable cytokinin application to reduce TSNA accumulation. The short term objective is 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 with a backpack sprayer at the rate of 50 gallons/acre, 27 ml/ plant (Figure 1), 24 hours before harvest. 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 four randomized complete blocks with four spray treatments and appropriate border rows, with some blocking for type of spray (cytokinin 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 2); 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 (Figure 1) 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 3) which was parked in the barn until housing four days after harvest (August 31st).

Sampling for molecular analysis

Samples for molecular analysis were taken from the railwagon the day after harvest (Figure 3). 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 4), 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 5).

Samples were placed on ice while a plot was being sampled (Figure 6), 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 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.

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.

Procedure – Molecular Laboratory

No molecular analyses were done, because cytokinin application did not significantly 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 (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 7). Figure 7 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 10C, 10D). 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 except midrib nitrate nitrogen (Table 1, Figures 8-10).

The high cytokinin rate significantly reduced midrib nitrate nitrogen relative to the low cytokinin rate, but neither was significantly different from the checks (Figure 10D). This is because of the blocking in the design, which allows a much more sensitive comparison within the main plots (between the two checks, and between the two cytokinin rates), than between the cytokinin treatments and the checks; accounting for the main plots takes out the variability within the main plots, thus reducing the

unexplained variability left in the error term. This is best illustrated in Table 2; the only comparison that is significant is cytokinin 1 vs cytokinin 2 (shown in red), because the error term for that comparison is the smallest.

One might speculate that in a season more conducive to TSNA accumulation, cytokinin application might have had a significant impact on reducing TSNAs.

Conclusions

In this one study, cytokinin application did not reduce TSNAs. However, it was a season very unfavorable for TSNA accumulation, and it is possible that in a more typical season, cytokinins 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.

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Figures and Tables

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

Constituent	Lamina Midrib	Transformation	*p* Value	Significance
NNN	Lamina	None	0.57	NS
NNN	Midrib	Log	0.68	NS
NAT	Lamina	None	0.46	NS
NAT	Midrib	Log	0.83	NS
Total TSNAs	Lamina	None	0.56	NS
Total TSNAs	Midrib	Log	0.69	NS
Conversion	Lamina	None	0.16	NS
Conversion	Midrib	None	0.064	NS
Total Alkaloids	Lamina	None	0.16	NS
Total Alkaloids	Midrib	None	0.30	NS
NO_2 N	Lamina	Log	0.39	NS
NO_2 N	Midrib	None	0.82	NS
NO_3 N	Lamina	None	0.45	NS
NO_3 N	Midrib	None	0.016	*
Total N	Lamina	Exponential	0.10	NS
Total N	Midrib	None	0.21	NS

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

Table 2: Treatments comparisons, showing differences of least squares means and standard errors

Differences of Least Squares Means							
Effect	**trt**	**_trt**	**Estimate**	**SE**	**DF**	**t Value**	**Pr > |t|**
trt	Check .	Cytokinin 1	-388.1	1,134.8	3	-0.34	0.755
trt	Check .	Cytokinin 2	1,010.4	1,134.8	3	0.89	0.439
trt	Check .	Water Check.	451.9	1,134.8	3	0.40	0.717
trt	Cytokinin 1	Cytokinin 2	1,398.5	174.1	3	8.03	0.004
trt	Cytokinin 1	Water Check.	840.0	1,134.8	3	0.74	0.513
trt	Cytokinin 2	Water Check.	-558.5	1,134.8	3	-0.49	0.656

Figure 1: Spray application with a backpack sprayer

Figure 2: Japanese beetles

Figure 3: Railwagon in barn

Figure 4: Leaf disc samples

Figure 5: Sampling pattern

Figure 6: Leaf disc samples on ice

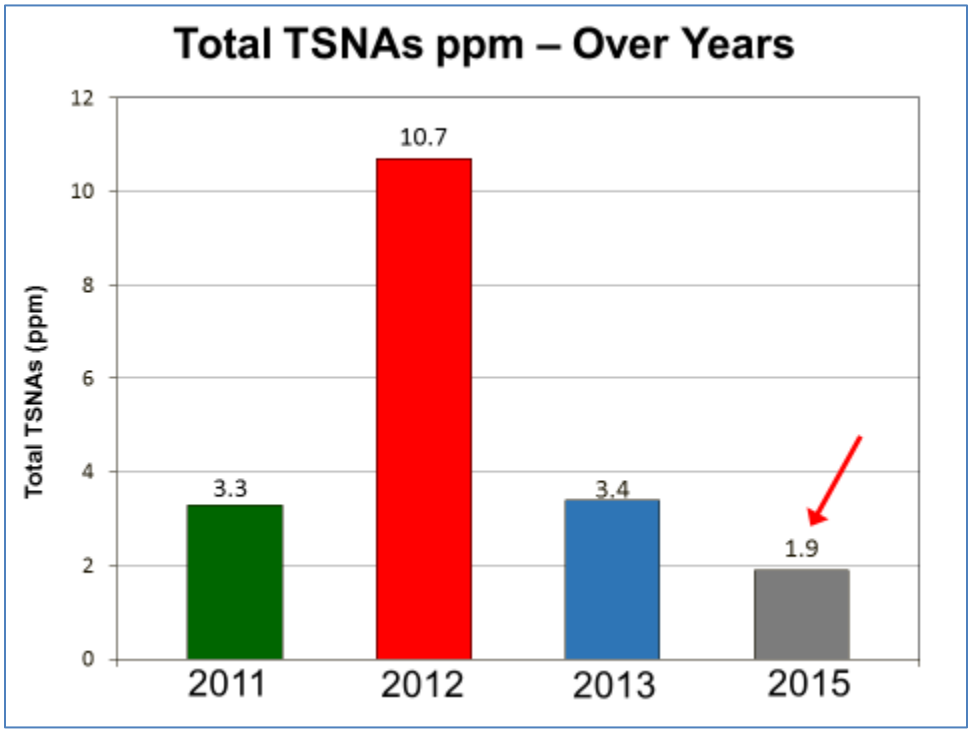
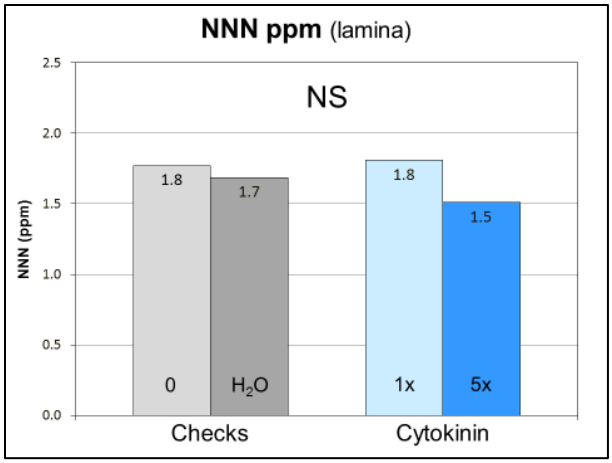


Figure 7: Total TSNAs in TN 90H transplanted in the last week of May, for the four years 2011 – 2015



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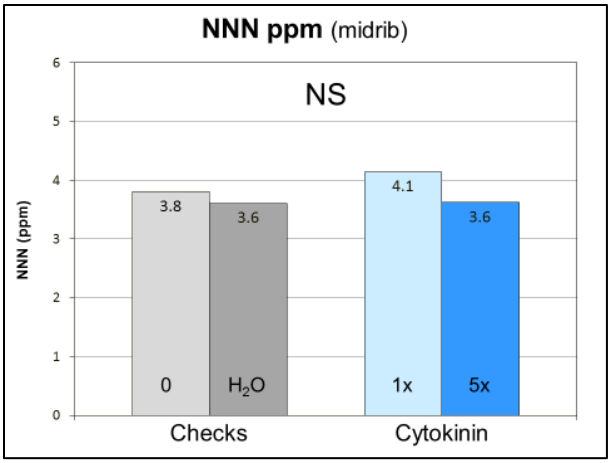

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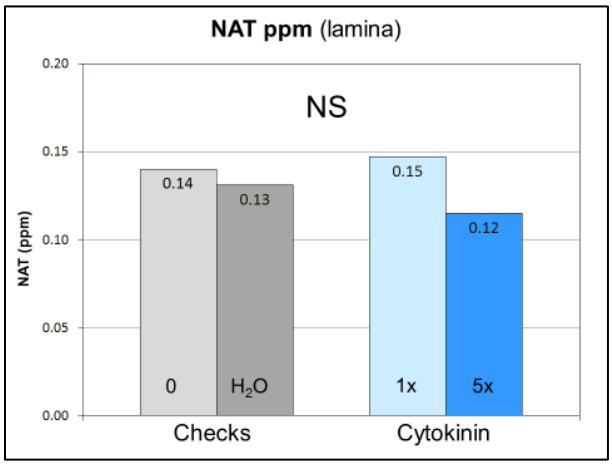

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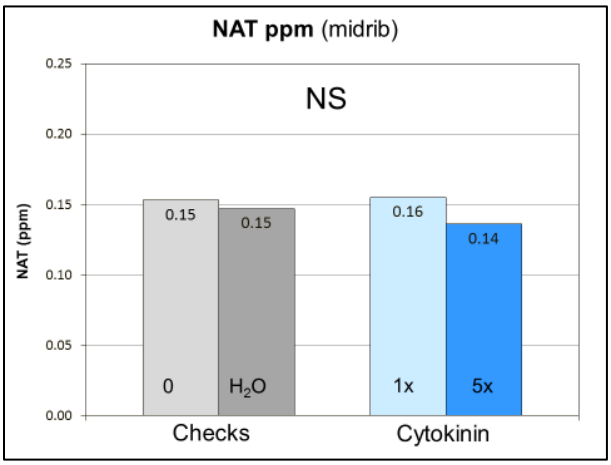

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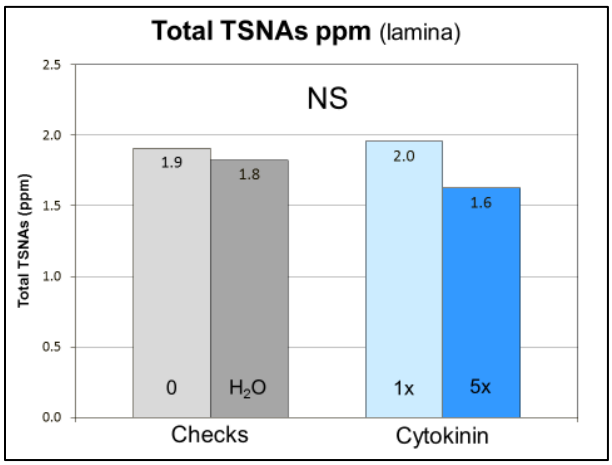

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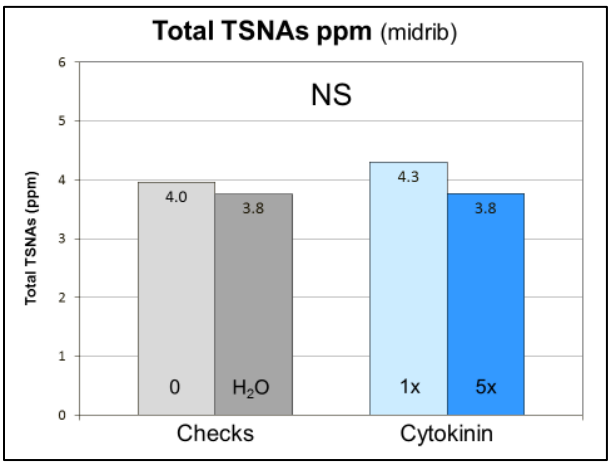

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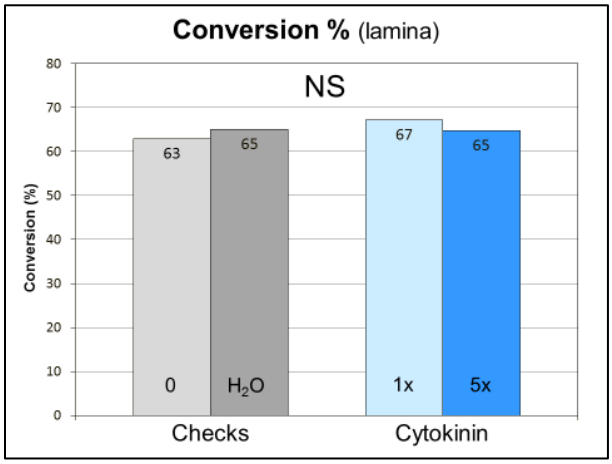
Figure 8: Effect of cytokinin sprays on TSNAs **A.** Lamina NNN **B.** Midrib NNN **C.** Lamina NAT **D.** Midrib NAT **E.** Lamina Total TSNAs **F.** Midrib Total TSNAs

NS = not significant (p>0.05)



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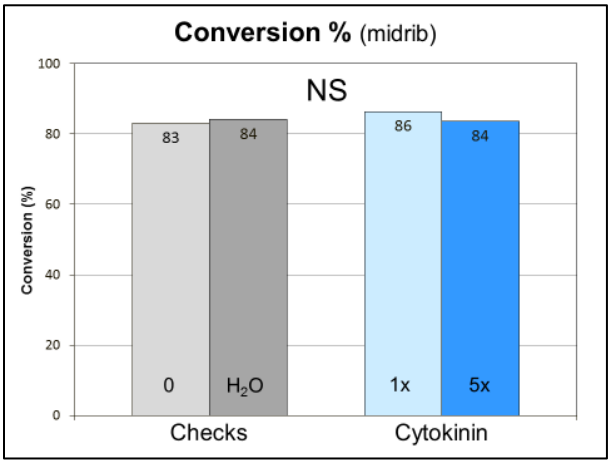

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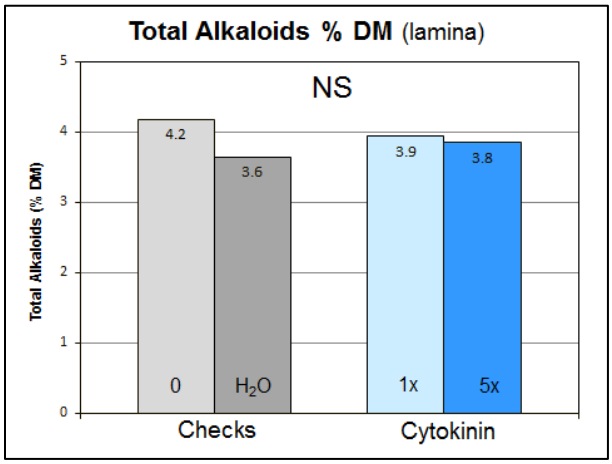

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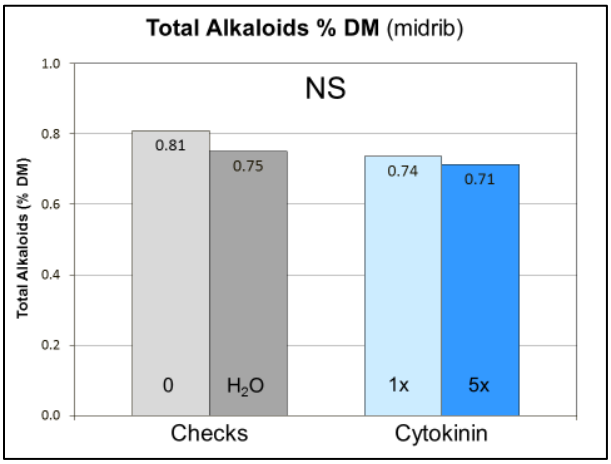

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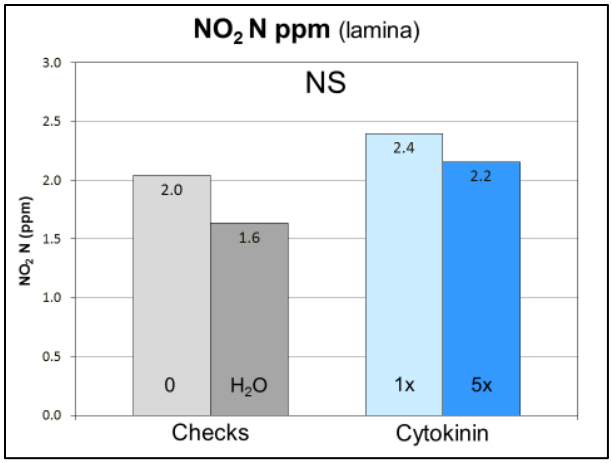
Figure 9: Effect of cytokinin sprays on alkaloids **A.** Lamina Conversion **B.** Midrib Conversion **C.** Lamina Total Alkaloids **D.** Midrib Total Alkaloids

NS = not significant (p>0.05)



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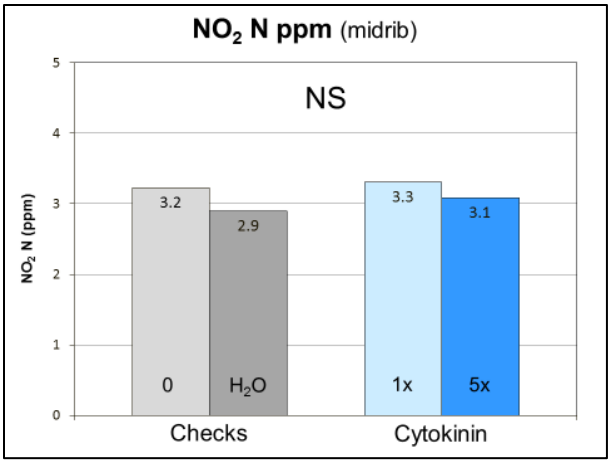

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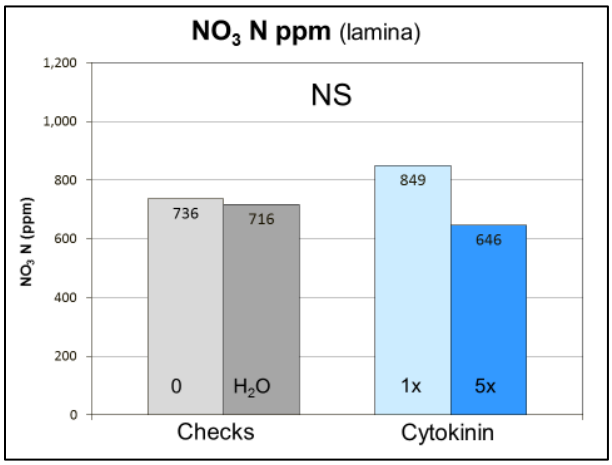

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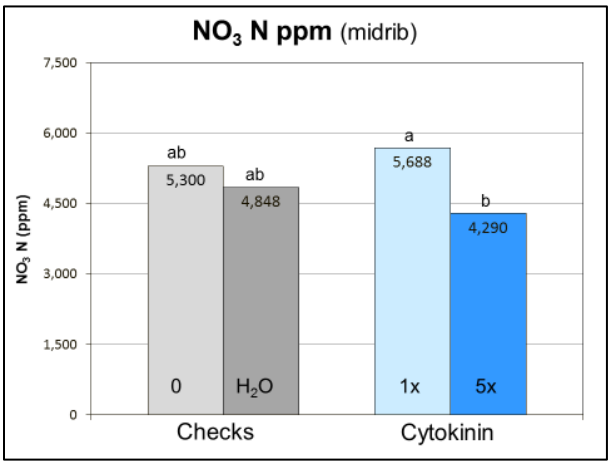

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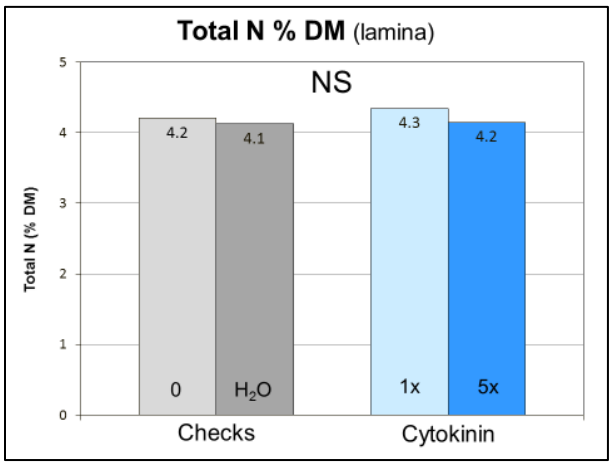

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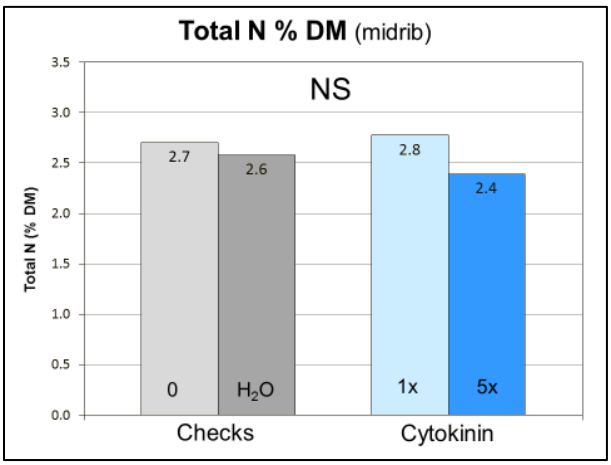

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Figure 10: Effect of cytokinin sprays on nitrogenous constituents **A.** Lamina NO_2 N **B.** Midrib NO_2 N **C.** Lamina NO_3 N **D.** Midrib NO_3 N **E.** Lamina Total Nitrogen **F.** Midrib Total Nitrogen

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