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

Title: **The Effects of Pre-Harvest Quercetin 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 naturally occurring flavonoid quercetin could be used to reduce TSNAs (Tobacco Specific Nitrosamines) in burley tobacco. Quercetin has antioxidant as well as antimicrobial properties, so in theory could affect TSNA accumulation. Plants were sprayed with quercetin the day before harvest, with a low (1x) and a high (10x) rate. There were no significant differences between the quercetin treatments and the controls, for any of the variables. There were significant differences between the quercetin rates for midrib NNN (N' nitrosonornicotine), midrib total TSNAs, lamina conversion and midrib total nitrogen; but 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, quercetin treatments may have an effect.

Introduction

Rationale

The goal of this study was to test whether spraying burley tobacco with the naturally occurring flavonoid quercetin leads to a reduction of tobacco-specific nitrosamine (TSNA) levels. Quercetin recently became a major focus of attention because of its reported health benefits, many of which are attributed to its antioxidant properties. Quercetin also has antimicrobial, antifungal and antiviral activity. Since both an increase in antioxidant capacity of tobacco prior to curing and a decrease in the plant-associated microbial population have been proposed as strategies to reduce TSNA accumulation, we hypothesize that a quercetin spray will reduce TSNA accumulation. 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 a simple spray method to reduce TSNA accumulation. The short term objective is to establish whether spraying burley tobacco with quercetin will reduce TSNA accumulation.

An effective chemical that would consistently reduce TSNA accumulation would be of enormous benefit to growers of air-cured tobacco and the tobacco industry.

Background

Flavonoids are a class of plant secondary metabolites with diverse functions in plants and animals (Middleton *et al.* 2000; Grotewold 2006; Petrussa *et al.* 2013). Similar to other secondary metabolites,

they are not essential for plant growth, but bring a competitive advantage to plants at certain developmental stages and under stress conditions. The molecular effects of flavonoids have been studied more in mammalian systems than in plants (Grotewold 2006; Ciz *et al.* 2012). In animals, flavonoids have been shown to have antioxidant properties, to modulate angiogenesis, regulate the cell cycle, to cause apoptosis and to affect several cancer-related biological pathways, including growth factor-mediated pathways, mitogen-activated protein kinase-dependent pathways, and ubiquitin/proteasome degradation pathways (Lila 2004; Lila 2007; Chen *et al.* 2008; Wiart 2013).

Quercetin is a flavonoid that belongs to the subgroup of flavonols (Figure 1). Two properties of quercetin are of particular interest for this project:

1. Direct antioxidant properties

Quercetin's antioxidative properties are due both to its direct scavenging of reactive oxygen species (ROS) and to its inhibitory action on some ROS-forming enzymes such as NADPH oxidase (Alegre *et al.* 2009; Ciz *et al*. 2012; Aboul-Enein *et al.* 2013; Brunetti *et al.* 2013; Bubols *et al.* 2013).

It has been postulated that TSNAs in cured leaves can be reduced by raising the levels of antioxidants in the tobacco leaves prior to harvesting the plants. Although it was reported that quercetin is taken up by plant cells and transported to other parts of the plant (Buer *et al.* 2008), these assays have not been conducted in tobacco. Internalized quercetin is a precursor for a number of other flavonoids (Winkel-Shirley 2001; Winkel-Shirley 2002; Winkel 2006). Most flavonoid species act as antioxidants (albeit to varying degrees), so even if all the quercetin molecules translocated into the tobacco leaves are metabolized, the antioxidative capacity of the cells would still be expected to increase.

2. Antimicrobial properties

The antibacterial activity of flavonoids has been recognized in the traditional medicine of many cultures, but has been investigated in more detail at the molecular level only recently (Cushnie *et al.* 2003; Cushnie and Lamb 2011). During TSNA formation, nitrite needed for the nitrosation of alkaloids is believed to be formed by bacteria residing in the tobacco leaves. We hypothesize that the antimicrobial activity of quercetin may affect the size of this bacterial population and thus lead to a reduced generation of nitrite and consequently TSNAs. The effects of flavonoids on foliar microbial population density and diversity have not been tested in tobacco.

Summary of Progress

Procedure – Field Work

Variety

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

Treatments

The treatments were two controls (water control and unsprayed) and two rates of an aqueous solution of quercetin. The water control and both rates of quercetin were applied with a backpack sprayer at 50 gallons/acre, 27 ml per plant (Figure 2), 24 hours before harvest. Since there are no reported studies using quercetin sprays on tobacco, we originally planned to test the effects of 1 mM and 10 mM

solutions. However, we found that we could not make a concentrated stock, so we reduced the concentration tenfold, to 0.1 mM as the low rate and 1 mM as the high rate. Like many compounds of biological origin, the rates of quercetin are very low; 0.2 and 2 oz/acre.

1. Unsprayed control
2. Water sprayed control, 50 gallons/acre, 24 hours before harvest
3. Low rate – 0.1 mM quercetin in 50 gallons/acre water, 24 hours before harvest
   30.2 mg/L, 0.004 oz/gallon, 0.2 oz/acre of product
4. High rate – 1 mM quercetin in 50 gallons/acre water, 24 hours before harvest
   302 mg/L, 0.04 oz/gallon, 2 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 (quercetin 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; 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 quercetin sprays and water control were applied with a backpack sprayer (Figure 2) 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).

The quercetin 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 4). 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 5), 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 6).

Samples were placed on ice while a plot was being sampled (Figure 7), 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.

<u>Procedure – Molecular Laboratory</u>

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

<u>Procedure – Analytical Laboratory</u>

Constituents analyzed

Both lamina and midrib were analyzed for all constituents.

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

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

Nitrate nitrogen

Nitrite nitrogen

Total nitrogen

Laboratory analysis

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

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

Results and Discussion

TSNAs and alkaloids were unusually low in Kentucky in 2015, as a result of the heavy early rain and consequent small root systems. Total TSNAs for the high converter TN 90H are typically over 10 ppm, but in the last five years, we have measured TSNAs over 10 ppm only once, in 2012 (Figure 8). Figure 8 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 11C, 11D). 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 the quercetin treatments and the controls, for any of the variables (Table 1, Figures 9-11). There were significant differences between the quercetin rates for midrib NNN, midrib total TSNAs, lamina conversion and midrib total nitrogen; but not between the quercetin sprays and the controls.

Both midrib NNN and midrib total TSNAs were lower in the 1x quercetin treatment than in the 10x (Figures 9B, 9F), contrary to expectation. This suggests that we should explore lower rates. Lamina conversion and midrib total nitrogen were lower in the 10x treatment than in the 1x (Figures 10A, 11F), as we expected. We do not yet have an explanation why the higher rate was more effective in reducing conversion and total nitrogen, but less effective in reducing TSNAs, but we will investigate this.

Although there was a trend towards lower TSNAs in the quercetin treatments compared with the checks, the differences were not significant, even though the much smaller differences between quercetin rates were significant. 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 quercetin rates), than between the quercetin treatments and the checks; accounting for the main plots takes out the variability between 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 quercetin 1 vs quercetin 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, quercetin application might have had a significant impact on reducing TSNAs, especially as there was a trend, albeit non-significant, towards lower TSNAs with quercetin application.

Conclusions

In this one study, quercetin 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, quercetin may be efficacious, especially as there was a trend, albeit non-significant, towards lower TSNAs with quercetin application.

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. We would also like to investigate further the effect of high vs low rates on TSNAs, conversion and total nitrogen.

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Figures and Tables

Table 1: Effect of quercetin 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) * significant (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 .	Quercetin 1	0.8475	0.3331	3	2.54	0.084
trt	Check .	Quercetin 2	0.5433	0.3331	3	1.63	0.201
trt	Check .	Water Check.	0.1959	0.3598	3	0.54	0.624
trt	Quercetin 1	Quercetin 2	-0.3043	0.0510	3	-5.97	0.009
trt	Quercetin 1	Water Check.	-0.6516	0.3598	3	-1.81	0.168
trt	Quercetin 2	Water Check.	-0.3473	0.3598	3	-0.97	0.406

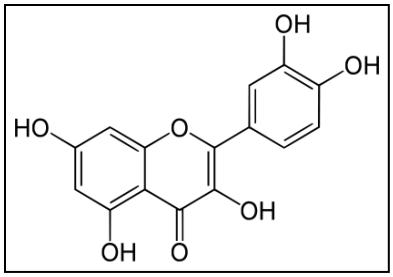


Figure 1: Structure of quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one)

Figure 2: Spray application

Figure 3: Railwagon

Figure 4: Taking samples for molecular analysis

Figure 5: Leaf disc samples

Figure 6: Sampling pattern

Figure 7: Leaf discs on ice, before being frozen in liquid N

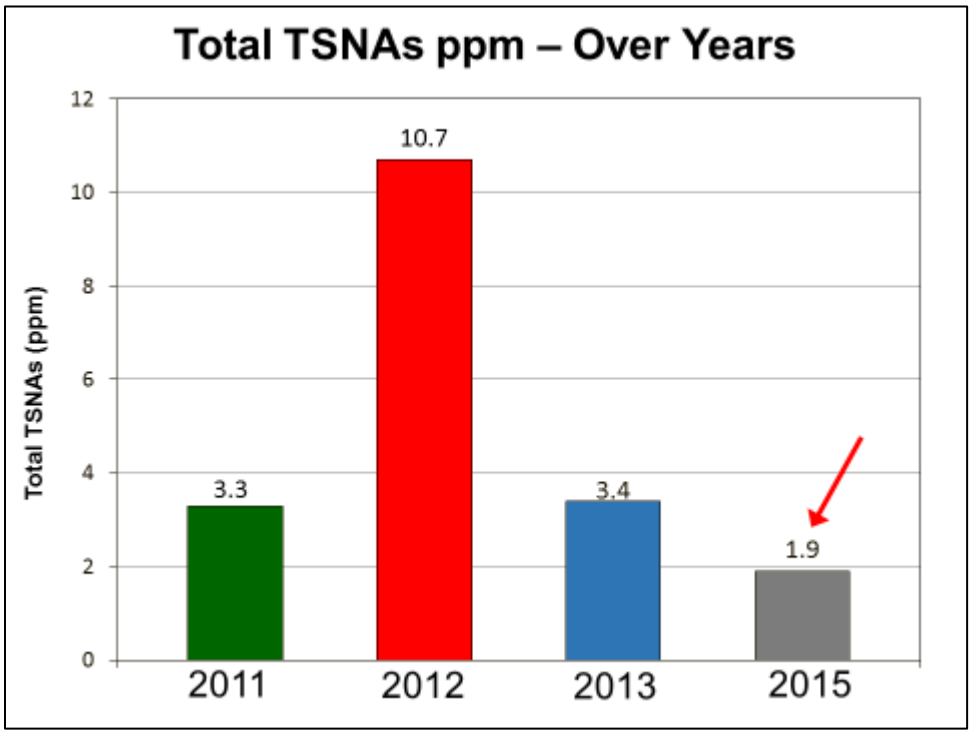
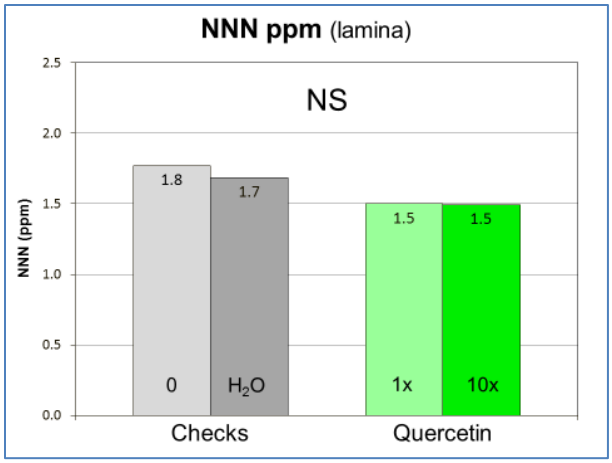


Figure 8: Total TSNAs in TN 90H transplanted in the last week of May, for the four years 2011 – 2015



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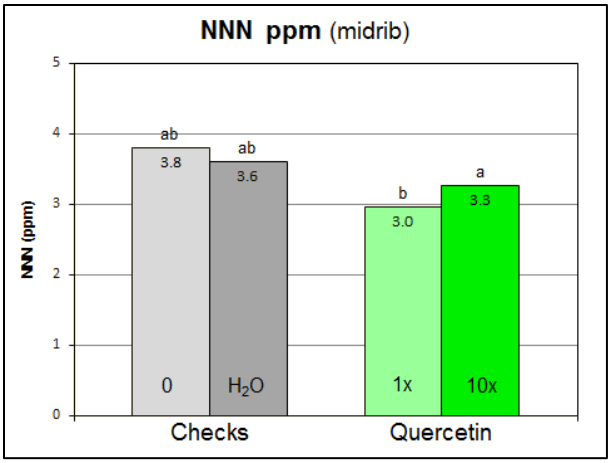

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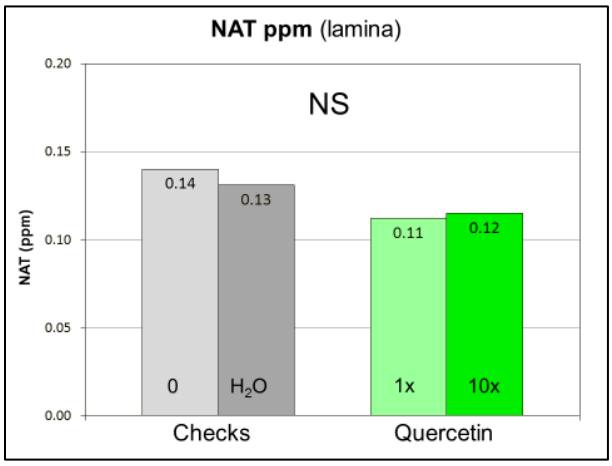

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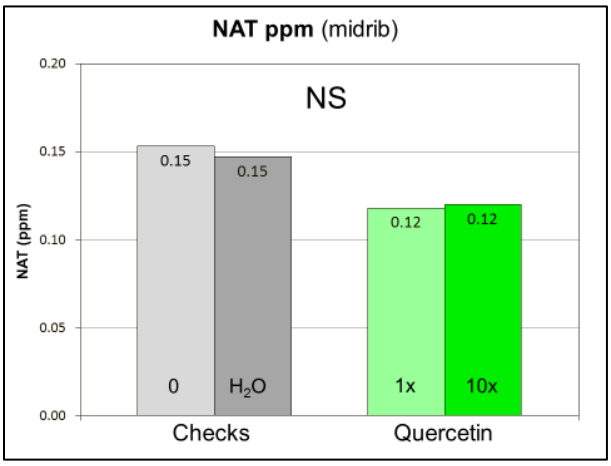

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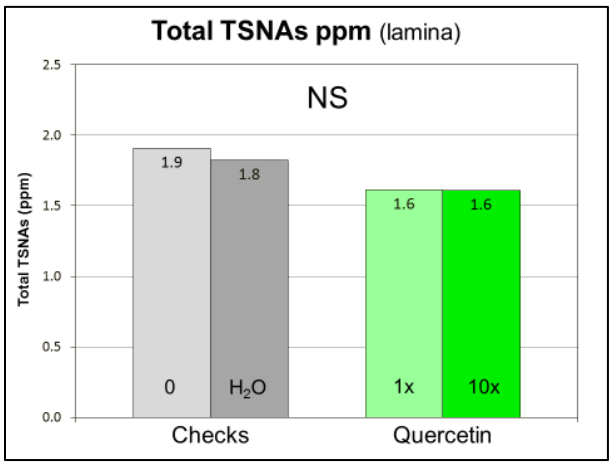

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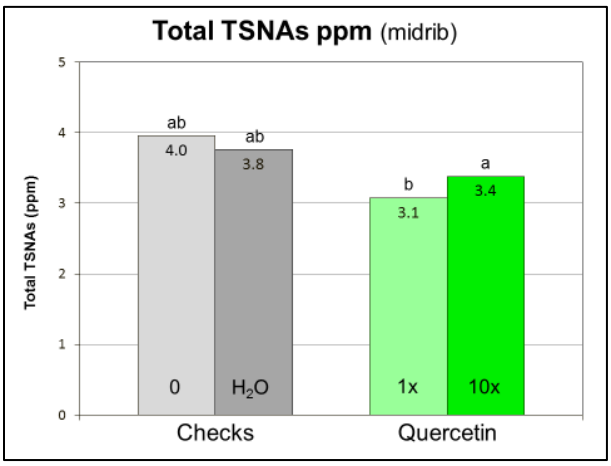

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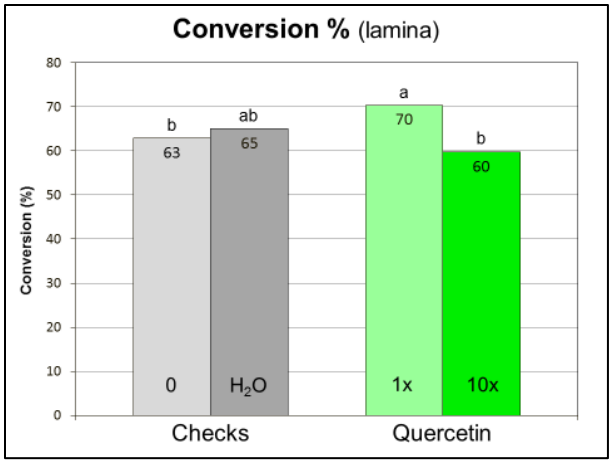
Figure 9: Effect of quercetin sprays on TSNAs **A.** Lamina NNN **B.** Midrib NNN **C.** Lamina NAT **D.** Midrib NAT **E.** Lamina Total TSNAs **F.** Midrib Total TSNAs

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



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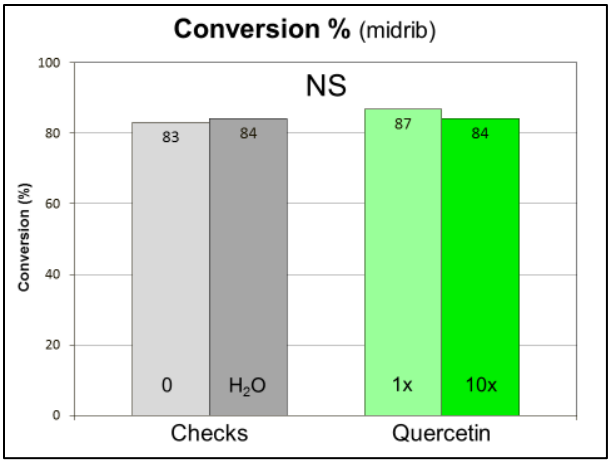

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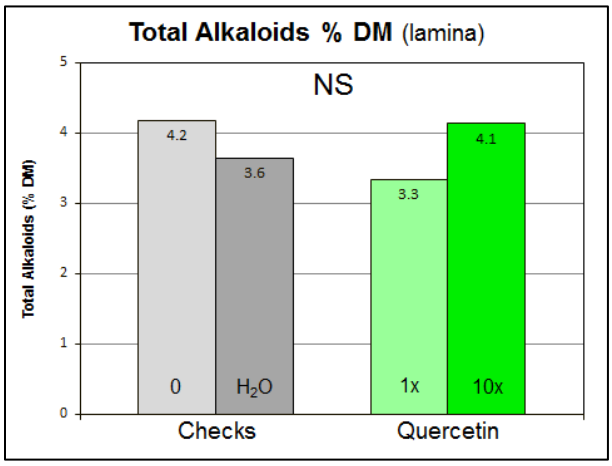

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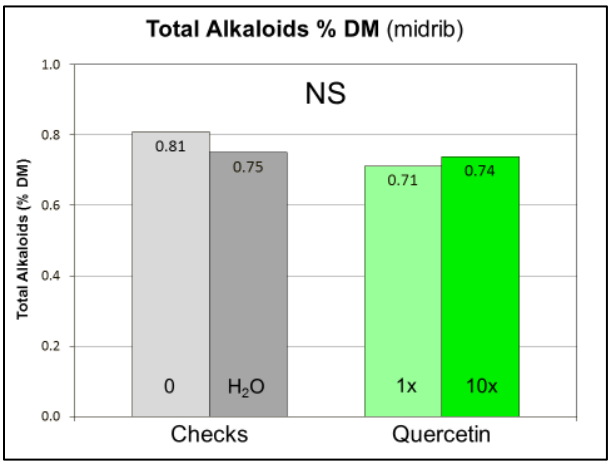

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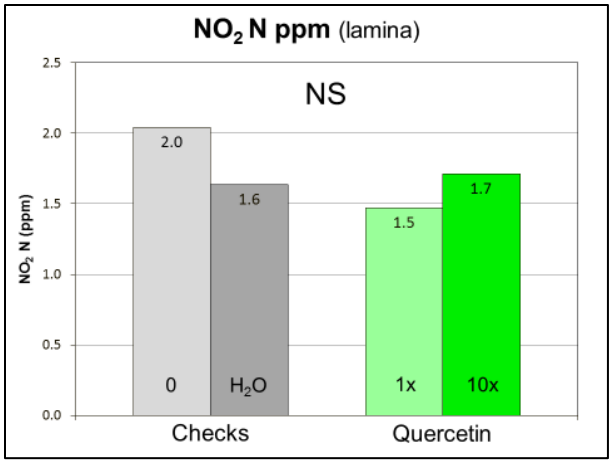
Figure 10: Effect of quercetin sprays on alkaloids **A.** Lamina Conversion **B.** Midrib Conversion **C.** Lamina Total Alkaloids **D.** Midrib Total Alkaloids

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



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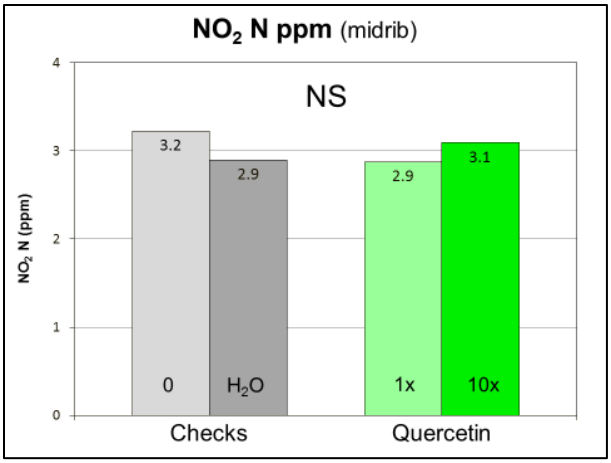

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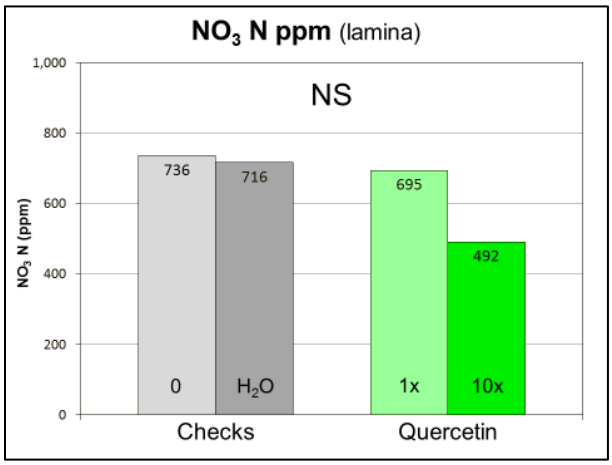

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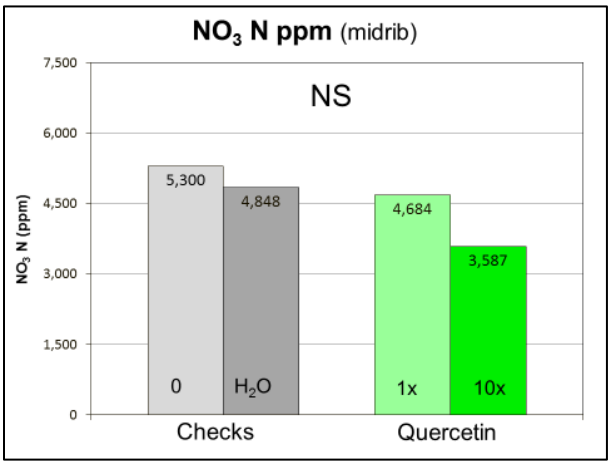

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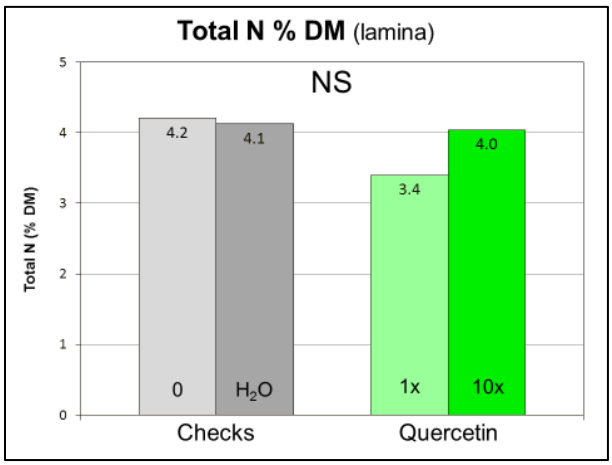

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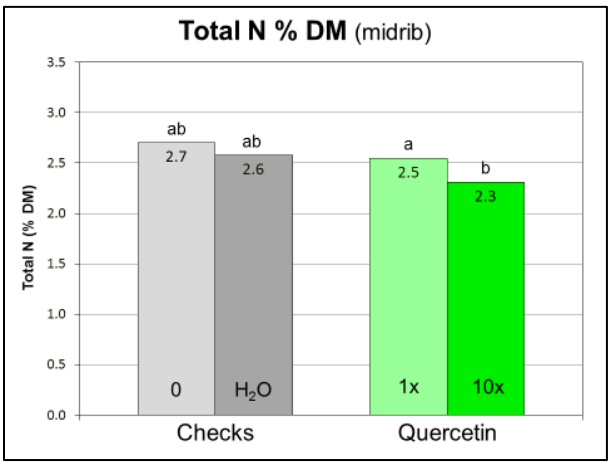

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Figure 11: Effect of quercetin sprays on nitrogenous constituents **A.** Lamina NO_2 N **B.** Midrib NO_2 N **C.** Lamina NO_3 N **D.** Midrib NO_3 N **E.** Lamina Total Nitrogen **F.** Midrib Total Nitrogen

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