

Effects of UV and Elevation on Flavonoid Production in Juvenile Landrace Maize Leaf Tissue

Research Thesis

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ABSTRACT

Many plant populations are locally adapted to conditions that vary across the landscape. At the maize center of diversity in Southern Mexico, maize landraces are locally adapted to the environments in which they grow. Maize from higher elevation zones has been selected under conditions of greater exposure to UV-B light compared to lower elevation landraces. This could be important as climate change urges farmers to plant varieties from warmer, lower climes at higher elevations with higher UV-exposure. Our goal was to understand the biochemical mechanisms that may maintain high performance under local conditions, and how this biochemistry changes under novel conditions. In this study, we investigated UV-B protective flavonoids produced by maize landraces collected at three elevations (600 m, 1550 m, and 2050 m) and planted in common gardens at 1550 m and 2050 m in Chiapas, Mexico. Using high performance liquid chromatography (HPLC), we examined the presence and quantity of maize leaf flavonoids maysin, quercetin-3-glucoside (q-3-glucoside), and quercetin-3-rutinoside (q-3-rutinoside) produced across 12 maize collections from three elevations (i.e. maize types) and garden locations. Due to results from a previous gene expression study and the likely adaptive importance of UV-B protection, we hypothesized that highland maize may have higher production of certain UV-B protective compounds than lower elevational types, with all types inducing greater production in response to increases in UV-B. Our results demonstrate that increases in elevation increased flavonoid production. This response was similar across maize types from different elevations, although highland types seemed to have a greater induced response. These results may have implications for maize production with climate change since moving of crop populations to higher elevations to address warming may be disrupted by differential UV-B adaptation. Future work could investigate the fitness effects on plants that

employ these different flavonoid pathways under higher and lower UV-B conditions to better understand desired chemotypes and their biological consequences.

INTRODUCTION

There are many environmental factors that, when plentiful or scarce, can create stressful conditions for plants, such as: temperature, resources (water, nutrients, light), soil conditions, and ultraviolet (UV) radiation. UV-B (280nm-320nm) radiation, which is constantly present in the environment but filtered by our atmosphere, may have significant negative effects on plant growth and can have destructive effects on a plant's physiology by altering metabolite production (Mewis et al 2012; Fagerberg and Bornman 2005). While decreases in earth's stratospheric ozone layer have increased UV-B infiltration for all plants, variation in UV-B can also be experienced across elevations (Ziska et al. 1992). Thus, UV-B may act as a selection pressure on plants, producing locally adapted populations that differ in their UV-B responses due to past selection.

The ability of a plant to deal with the presence of, or changes in, UV-B radiation depends in part on the induction of UV-B protectant compounds. No matter the fluence rate of UV-B, plants will upregulate flavonoid production as an induced response to the addition or/increase of UV-B radiation. If there is a high fluence rate, then plants may also create an antioxidant defense system, (Jenkins and Brown 2007). Production of flavonoids constitutes an important induced response for plants to deal with the stresses produced by UV-B. Flavonoids are known UV-B protectants in maize and other plants and are stored in the vacuoles of epidermal plant cells, where 90-99 percent of UV-B absorption occurs (Casati and Walbot 2005; Stafford 1991). This upregulation of flavonoids can be seen as a form physiological plasticity that helps plants adjust

to changing environmental conditions, such as climate change (Gratani 2014). Therefore, investigating the physiological plasticity of populations and elevational types is important to understand how plants adapt to variable levels of UV-B radiation. It can also have relevance to questions of how plants respond to overall increases in UV-B due to atmospheric shifts.

Maize has been a study system for understanding the biosynthesis of these protective flavonoids since the spread of maize across elevations from its low latitude, center of origin, attests to its ability to adapt to varying UV-B levels (Casati and Walbot 2003; Perales and Golicher 2014). Based on the literature populations from low latitude and high elevations can be expected to be those best adapted to higher UV-B conditions and be able to produce larger amounts of these UV-B protectant flavonoids (Ziska et al 1992; Dahlback et al 1989). Studying local adaptations to UV-B across a large elevational gradient is important to understand the constitutive and induced responses to UV-B because populations growing at higher elevations must be able to tolerate the higher levels of UV-B present in those areas; populations inhabiting lower elevations must tolerate less (Jansen et al. 1998).

Most work on flavonoid production in plants has been performed only with plants from higher elevations due to their putative UV-B protectant adaptations (Ziska et al 1992). Researchers have studied flavonoid response in highland types (mainly). Not much previous work has been done studying constitutive and environmentally induced responses through the production of UV-B from populations from lower elevations. Additionally, most have manipulated responses to UV-B under controlled conditions (greenhouses and growth chambers) through the use of UV lamps and/or cellulose acetate filters (Ziska et al 1992; Casati and Walbot 2005). Few have investigated this response using field conditions under which UV-B is expected

to differ. This study focuses on studying flavonoid production in different locally adapted types across an elevational gradient under high and low UV-B field conditions.

Previous work has informed our expectations for our various maize elevational types in response to UV-B and specifics of the UV-B protectant compounds that may differ. Prior genetics work in our lab using plants grown at mid-range elevations (1550m) demonstrated that there was an upregulation in the RNA expression of genes important in the production of flavonols in highland types (i.e., populations from 2050 m) compared to lowland types (i.e., populations from 600m); there were also no significant differences in the upregulation between midland types (populations from 1550m) and highland types (from 2050m; Kost 2014). Thus, there may exist a genetic difference or differential constitutive responses in flavonol production between types from different elevations. Kost (2014) found that quercetin-3-glucoside (q-3-glucoside) was part of the flavonols for which gene expression differences might have increased production in maize from higher elevations (see Fig 3.5 in Kost 2014). Quercetin-3-rutinoside (q-3-rutinoside), a quercetin derivative similar to q-3-glucoside, is commonly found in nature and was also considered in this study (Solimani 1997). In other biochemical work, researchers have shown that, when exposed to high UV-B, maize from higher elevations increases production of the flavone maysin, a compound that makes up to two percent of biomass in silks, and its precursor rhamnosylisoorientin (Casati and Walbot 2005). For this thesis, we looked specifically at concentrations of three UV-B protectant compounds: one flavone, maysin, and two flavonols, q-3-glucoside and q-3-rutinoside. Other compounds such as chalcone and kaempferol have been seen to be important UV-B protectants and have been previously studied, but are not included in this study.

In order to investigate the genetic and environmental factors affecting production of flavonoids in maize in Chiapas, we collected maize populations from different elevations (600 m, 1550 m, and 2050 m) and grew them at mid (1550 m) and high (2050 m) elevations. We reasoned that highland types would have a greater production of flavonoids in both gardens compared to that of maize from lower elevations. We also expected that midland and highland types may have similar constitutive responses. We expected that the induced response by all types would be similar; all types would increase production of flavonoids with an increase UV-B radiation. Investigating the difference in production of these flavonoids in two different locations will help us understand the degree to which the biochemistry of each type results from constitutive and environmentally induced responses.

METHODS

Experimental Design

Chiapas is a hotspot of diversity for maize in Mexico (Perales and Golicher 2008). This large diversity stems from a large cultivation range (0-2600m) of landrace maize and the varying conditions along the elevational gradient, including changes in rainfall, soil type, irradiance, temperature, and UV-B (Perales and Brush 2007). In 2009, we visited three farmers in each of three different towns at each of three elevational ranges: lowland elevation (563-648m), midland elevation (1533-1584m), and highland elevation (1941-2153m). Therefore, a total of 27 populations were collected from these local farmers, nine populations of each elevational type. Twelve of these 27 populations were then chosen for this study, four populations from each of the three elevations (Table 1.). Populations were composed of the seeds from 50 whole ears

from a landrace seed lot that had been cultivated by the farmer for 10+ years. These populations were then planted in a reciprocal transplant common garden experiment at two elevations (Pace in preparation). The highland garden was planted in Chichihuistán (2056 m, 16°35'56.06" N 92°33'41.06" W) and the midland garden was planted in La Independencia (1523m, 16°14'30.02" N 92°00'46.44" W); a third lowland garden was planted in Quespala (600 m, long. 15°81'50.00" N 91°94'77.78" W) but tissue samples were unusable. At each planting site we used a modified split-split plot design with three blocks. In each block, plants were separated by elevation type (i.e., three main plots) in order to limit any phenology or height effects by neighboring individuals from different elevation types. Each main plot was divided into subplots by population, with four populations per main plot. In each subplot we had four rows, each with seven individuals sown in traditional fashion, three seeds per planting location or "*mata*" with *matas* and rows were separated by one meter. In addition, border *matas* were planted around sub and main plots in order to limit edge effects and destructive sampling effects on neighboring subplots. Since flavonoid content in landrace maize leaf tissue is greatest at the juvenile stage, after six weeks the third mature leaf was collected from randomly selected *matas* and lyophilized, to obtain three leaf samples from three individuals of each population (Casati and Walbot 2005). In total, we collected 36 leaf samples per block per garden were harvested.

High Performance Liquid Chromatography (HPLC)

Leaf tissue was frozen in liquid nitrogen and ground three times using a mortar and pestle until only a fine powder remained. 0.0100g ± 0.0005g was weighed and used for extraction in 3 mL of acidified methanol (1% HCL in methanol) and left over night. 10µL of known concentration of hesperetin was added to each sample to normalize the results. Hesperetin was chosen as the

internal standard for normalization because it is a known flavonoid of other plants, with a peak absorbance of 350nm that is not expected in juvenile maize leaf tissue. Samples were centrifuged 3 min at 3000 x g. The supernatant was filtered and 1 mL was loaded onto high-performance liquid chromatography. Injecting 10µl into a W2695 Waters separation module and a W2996 photodiode array (PDA) using a Symmetry C18 reverse column(3.5 µL, 4.9mm, 75mm, Waters). Separation occurred with a flow rate of 1.0mL min⁻¹ for 30 min while using 5% acetic acid and acetonitrile as solvent A and solvent B respectively, as follows: gradient of 20 min 95% solvent A and 5% solvent B, 2 min 65% solvent A and 35% solvent B, 0.10 min 20% solvent A and 80% solvent B, 4 min 100% solvent B, 3.90 min 95% solvent A and 5% solvent B. Ninety-five percent of solvent A and five percent solvent B was chosen after a trial run with 90% solvent A and 10% solvent B showed lower concentrations of peaks than expected. Samples were run through HPLC along with external standards of q-3-glucoside, q-3-rutinoside, maysin, and hesperitin. Data was recorded and analyzed at known flavonoid wavelength of 350nm using Empower 3 software. Other known flavonoid wave lengths had to be done separately so only compounds that have peak absorbance at 350nm were used. Peaks were identified using external standard retention times.

Data Analysis

Normalization, creation of standard curves, and analysis of peaks were performed with R-studios 3.2.5. Using lme4 package, we were able to create a generalized linear mixed model to analyze the factors influencing the concentrations of our three flavonoids. Any samples that had an unknown concentration of internal standard and created a poor normalization constant were

removed. More interestingly, any sample that did not produce a particular compound was removed from the analysis of that compound and we summarized the data on the number of samples lacking particular compounds. Our model included the fixed effects of garden, elevational type (known as type), and the garden by type interaction, as well as the random effects of block and population within type and the interaction between garden and type. We determined the significance of these factors using analysis of variance (with type III sums of squares) and with Satterthwaite approximations for our degrees of freedom. Least squares means and standard errors were presented.

RESULTS

Overall, we found a significant increase in q-3-glucoside production as elevation increased (Table 2). For this induced response, there was almost a two-fold increase by all types in production of q-3-glucoside under higher UV-B conditions (Fig 1). Although midland and highland types both produced more q-3-glucoside in the midland and highland garden than lowland types, our results show no significant differences among types for production of q-3-glucoside, contrary to our expectations. While it appears that the highland type might have increased its q-3-glucoside production slightly more than midland or lowland types (Fig 1), this interaction was not significant (Table 2).

Q-3-rutinoside was the compound in each of the two gardens that was produced in greatest quantities (Fig 1, 2, and 3). Although, not significant, there seems to be a trend of increasing q-3-rutinoside production by all types as elevation increases ($p = 0.151$). Production

by the highland type was least in the midland garden, but largest in the highland garden, resulting in an apparently greater induced response than seen in the other types; both the midland and lowland types had similar induced responses (Fig 2). Nevertheless, the interaction was not significant (Table 3). This analysis supports the existence of a similar constitutive response across types.

There was no significant effect garden for maysin, however, the relatively low p-value ($p = 0.1071$), may mean the trend of increasing production at higher elevations could be of interest (Tables 2, 3, and 4). Neither the effect of type nor the interaction were significant either. Nevertheless, we do see that maysin production increased somewhat more in highland types with increases in UV-B exposure than it did in midland or lowland types.

Overall, for individuals that produce them, there appeared to be induced effects by all types when placed at higher elevations, supporting the literature that there is higher production of flavonoids as elevation increases. Nevertheless, only analyses of q-3-glucoside shows clear evidence of induced responses. Interestingly, in all three compounds, highland types seem to have the largest increase in flavonoid production as elevation increased, although neither type nor interaction of garden and type were significant. Thus, while a similar constitutive response across types is supported in two compounds (maysin, q-3-rutinoside) due to the lack of significance for type or garden, some additional interesting trends do exist (Figures 1, 2, and 3; Tables 2, 3, and 4).

Despite the trend of increased flavonoid production as an induced response to higher UV-B radiation in the highland garden, there were seven individuals that failed to produce one of the compounds looked at in this study. All seven missing peaks came from seven separate

individuals. Many of the individual plants that failed to produce one of these compounds were in the highland garden, in particular (Table 5) There was a lack of production of q-3-rutinoside by five individuals (four of which were planted in the highland garden), a lack of production of maysin in two individuals (one of which were planted in the highland garden). In total, five of the seven compounds that were missing came from individuals from the highland garden (Table 5).

DISCUSSION

Flavones and flavonols are compounds that play a vital role in maize physiology and protection from UV-B radiation (Kost 2014; Jaakola and Hohtola 2010). Highland farmers have likely long relied on adaptation to UV-B for maintaining production. Our results indicate that environmentally-induced flavonoid production may be an important part of that adaptation, in some cases. However, due to variability in our data, it is hard to rule out the possibility that constitutive production across elevation of origin, may dominate.

There are two ways that our work builds on the current literature of the subject of biochemical responses to UV-B. First, most research investigates flavonoid production in response to controlled changes in UV-B (Casati and Walbot 2005). For instance, many have used UV-B lamps and cellulose filters to control the amount of UV-B and overall radiation. However, to understand what kinds of responses are common under environmental conditions that shift across the landscape, it is essential to use field studies. In this work, we looked at the production of flavones and flavonols across a natural elevational gradient in field experiments. This allowed for more realistic and applicable results for farmers of landraces. However, having no environmental control means that we may be confounding UV-B levels that increase with

elevation with other biotic and abiotic factors that may also shift with elevation such as temperature, rainfall, and microbiome diversity among others (Wang et al. 2012; Lipp et al. 1994). Plus, the use of a field experiment means that we were assaying responses across variable microclimates within each common garden, which may have led to the variability in flavonoid production. While we saw induction of greater amounts of flavonoid production (by those that produced it) under higher elevations, five of the seven missing cases where a particular compound was not produced were from the highland garden. This may suggest that microclimates may have had a larger effect on individuals from the highland garden and their production of compounds.

Second, highland populations are widely believed to produce more UV-B protectant flavonoids as a result of being adapted to higher UV-B environment. Therefore, highland types have been the focus of flavonoid production experiments (Ziska et al. 1992; Casati and Walbot 2005; Bieza and Lois 2001). In fact, there has not been previous comprehensive study on induced response on flavonoid production across different natural environment conditions from maize that is expected to be differentially adapted (i.e., our elevational types). This study is one of the first to look at the production of these important secondary metabolites across different UV-B environments from populations adapted to environments below 2000 m. Yet, we did not find significant differences among the responses of our types, contrary to previous expectations, although we did see a consistent trend with the highland type increasing production more with elevation than the other types. The use of landraces of maize means that we assayed materials with significant genetic variation within type and within population. That genetic variation and variation in UV-B response that may accompany that variation that may have obscured responses.

We were only able to report here on three compounds, which can only provide part of the picture of how maize seedlings respond biochemically to different environments. Investigating more compounds and discerning a chemotype for each elevational type may provide for a better understanding of the dynamics of flavonoid production in different UV-B environments. Further investigation of the production of other UV-B protectant flavonoids such as quercetin, kaempferol, chalcone, and others will help identify type-specific or population-specific chemotypes and how they shift with elevation. This will provide insight on the preferred secondary metabolites for differing UV-B conditions and environments.

Our results demonstrate that increased flavonoid production may be induced in response to changes in environment from midland to highland gardens for some compounds. This induced increase was significant for q-3-glucoside, but not for maysin and q-3-rutinoside. Although we saw no significant interactions between type and garden, further research with greater control on environmental or genetic variation, may clarify whether the different types increased flavonoid production to the same degree as elevation increased. Also our results confirm previous RNA sequencing work that provided the expectation that production of q-3-rutinoside and q-3-glucoside might be similar between highland and midland types in the midland garden, since we found a lack of significance between types in the midland garden. Yet, those type were not shown to differ from the lowland type. Our results also suggest that highland types may have a greater ability to alter their secondary metabolite production in response to varying UV-B conditions. This may be due to larger variation in UV-B in their locally adapted climates or because they have adapted to larger amounts of UV-B, in response to which they can produce a larger amount of UV protectants than lower types. Again, investigating more compounds across

this elevational gradient by different types may provide more insight on the ability of types to deal with rapid changes of UV-B.

The individuals that failed to produce maysin were of lowland origin. Previous studies have shown an increase in the production of maysin by highland types with increased stress caused by UV-B (Casati and Walbot 2005). The lack of production by these individuals may demonstrate that maysin is more preferred by highland types, which are exposed to higher UV-B conditions. At the same time, when individuals from non-highland types (from < 2000 m) do produce maysin they are able to produce the same if not more of the compound than highland types. Thus, these missing compounds may be due to capturing unidentified precursors or other error, rather than a relevant biological phenomena.

Investigation of genetic factors may help clarify the production of this compound by being able to determine how much of the production is due to genetic difference and how much can be attributed to induced response of higher UV-B conditions. Highland types were seen to trend towards having greater induced responses (Figures 1, 2, and 3.) compared to lowland and midland types. With rapid changes in climate phenotypic plasticity could be an important survival then genetic plasticity (Gratani 2014).

We see some increases in flavonoid production in some compounds with increasing elevation. While we can correlate elevation with flavonoid production and assume that higher flavonoid production is better for plants under high elevation, we cannot directly identify the effects of increased flavonoids on plant fitness from this data. Investigating the fitness effects of different chemotypes across this elevational gradient will allow us to determine which flavonoids, if any, have the greatest impact on UV-B protection.

UV-B has been proven to have destructive physiological effects on plants and with increasing UV-B radiation reaching the earth's surface, flavonoid production in the leaf tissue of maize, which accounts for almost all of the UV-B absorption, becomes a more and more important response for maize (Casati and Walbot 2005; Stafford 1991). Failure to respond adequately to changes in UV-B, by producing more flavonoids, may have detrimental effects on overall plant fitness. Phenotypic plasticity and the ability of a plant to respond to rapid changes in conditions also contributes significantly to plant fitness. Investigating more UV protectant compounds such as quercetin, kaempferol and its glucosides, and chalcone will allow for a more comprehensive understanding of phenotypic plasticity in terms of flavonoid production and its overall impact on fitness.

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TABLES

Table 1. Population, cultivation, elevation, and location data for all populations of landrace maize collected and planted in Chiapas, Mexico. Four of the nine populations were selected in each elevational type (LL = lowland, ML = midland, HL = highland).

Type	Population #	Municipality	Community / Company	Elev	Lat	Long	Race
LL	1	Chicomuselo	Raizal	648	15.894	92.254	Tuxpeño
LL	4	Frontera Comalapa	Benito Juárez	563	15.823	92.204	Tuxpeño
LL	7	La Trinitaria	Juan Aldama	598	15.855	91.938	Tuxpeño
LL	9	La Trinitaria	Nuevo Llano Grande	595	15.839	91.936	Tuxpeño

ML	10	La Trinitaria	El Rosario Tierra Blanca	1533	16.077	- 91.746	Comiteco
ML	12	La Trinitaria	Miguel Hidalgo	1524	16.106	- 91.778	Comiteco
ML	13	Comitán de Domínguez	San Francisco El Ricón	1584	16.281	- 92.136	Comiteco
ML	17	Las Margaritas	Ignacio Zaragoza	1531	16.352	- 91.919	Comiteco
HL	20	Comitán de Domínguez	Santa Elena Las Agujas	2089	16.361	- 92.179	Olotillo
HL	26	San Cristobal de Las Casas	Carrizal	2153	16.671	- 92.654	Olotón
HL	29	Teopisca	San Isidro Chichiuistán	1941	16.598	- 92.566	Olotón
HL	30	Teopisca	San Isidro Chichiuistán	2060	16.602	- 92.559	Olotón

Table 2. Analysis of Variance (ANOVA) table for fixed and random effects on production of quercetin-3-glucoside.

Fixed Effects	NumDF, DenDF	F-Value	P-value	
Garden	(1, 6.0240)	8.0824	0.02932	*
Type	(2, 64.165)	1.1074	0.33664	
Garden x Type	(2, 64.165)	0.0859	0.91783	
Random Effects	DF	Chi.Sq	p-value	
Population(Type)	2	0	1	
Block(Garden)	2	0.7344	0.7	
Type x Population x Garden	1	2.84E- 14	1	

Table 3. Analysis of Variance (ANOVA) table for fixed and random effects on production of quercetin-3-rutinoside.

Fixed Effects	NumDF, DenDF	F-Value	P-value
Garden	(1, 6.1723)	2.68544	0.151
Type	(2, 24.3391)	0.84957	0.4399
Gardne x Type	(2, 24.3391)	1.00255	0.3816
Random Effects	DF	Chi.Sq	p-value
Population(Type)	2	2.84E-14	1

Block(Garden)	2	8.06	0.02	*
Type x Population x Garden	1	3.17E-01	0.57	

Table 4. Analysis of Variance (ANOVA) table for fixed and random effects on production of maysin.

Fixed Effects	NumDF, DenDF	F-Value	P-value	
Garden	(1, 6.040)	3.5773	0.1071	
Type	(2, 61.106)	1.2392	0.2968	
Garden x Type	(2, 61.106)	0.29696	0.7646	
Random Effects	DF	Chi.Sq	p-value	
Population(Type)	2	0	1	
Block(Garden)	2	15.6	4.00E-04	***
Type x Population x Garden	1	0.00E+00	1	

Table 5. Proportion of individuals that failed to produce either q-3-glucoside, q-3-rutinoside, or maysin over the total individuals by garden and by type for each compound. 8 individuals (.0375 %) failed to produce one of the three compounds but did produce the other two.

Garden	Midland			Highland			Total
	Lowland	Midland	Highland	Lowland	Midland	Highland	
Quercetin-3-Glucoside	0/11	0/12	0/12	0/12	0/12	0/12	0/71
Quercetin-3-Rutinoside	0/11	0/12	1/12	0/12	1/12	3/12	5/71
Maysin	1/11	0/12	0/12	1/12	0/12	0/12	2/71
Total	1/33	0/36	1/36	1/36	1/36	3/36	7/213
	2/105			5/108			

FIGURES

Figure 1. Production of q-3-glucoside (μg) in response to growth at different elevations, differing in UV-B-exposure. At both the lower UV-B-exposed midland garden (1500 m) and the higher UV-B-exposed highland garden (2050 m), we planted three different locally adapted elevational types: lowland (600 m), midland (1500 m) and highland (2050 m). Least squares means (with standard errors) on q-3-glucoside production by those that produced the compound.

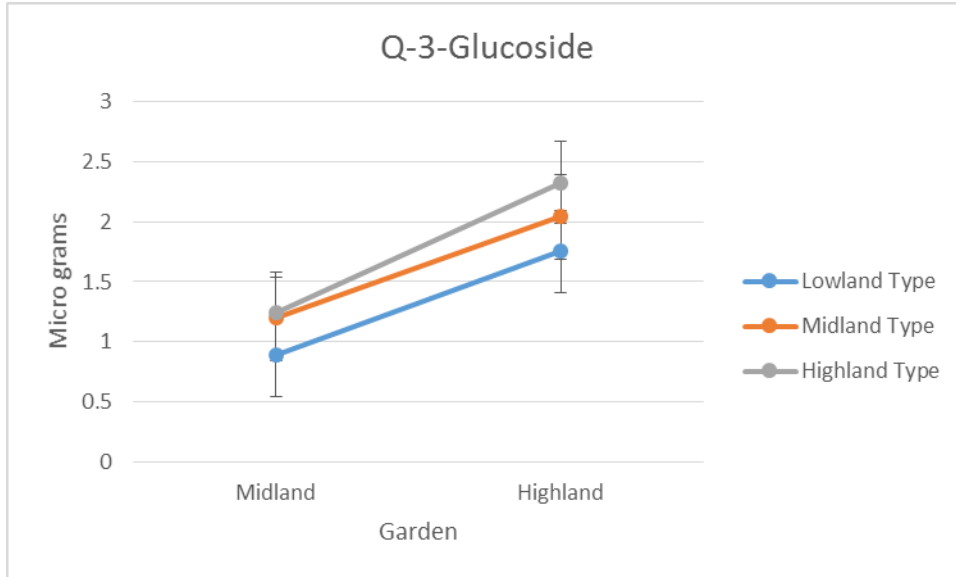


Figure 2. Production of q-3-rutinoside (μg) in response to growth at different elevations, differing in UV-B-exposure. At both the lower UV-B-exposed midland garden (1500 m) and the higher UV-B-exposed highland garden (2050 m), we planted three different locally adapted elevational types: lowland (600 m), midland (1500 m) and highland (2050 m). Least squares means (with standard errors) on q-3-rutinoside production by those that produced the compound.

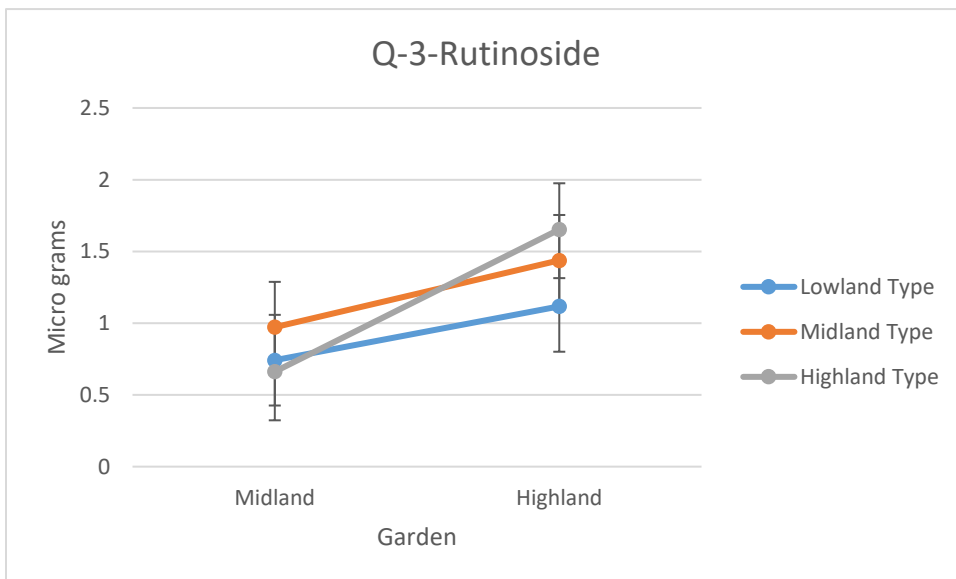


Figure 3. Production of maysin (μg) in response to growth at different elevations, differing in UV-B-exposure. At both the lower UV-B-exposed midland garden (1500 m) and the higher UV-B-exposed highland garden (2050 m), we planted three different locally adapted elevational types: lowland (600 m), midland (1500 m) and highland (2050 m). Least squares means (with standard errors) on maysin production by those that produced the compound.

