Determination of Specific Leaf Areas Among Differing Aspen Genotypes Along a Gradient of Nitrogen Conditions

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In this project I assess the specific leaf area (leaf area per unit of dry leaf mass) for 10 aspen genotypes from the Swedish Aspen (SwAsp) collection, which are grown under a range of different nitrogen conditions in a common garden in Kulbäcksliden. The primary questions leading this inquiry include: (*i)* does genetic variability have any effect on specific leaf area (SLA), (*ii*) in what ways does a gradient of nitrogen conditions affect SLA, and (*iii*) what is the combined effect of the interaction between the genotype and the environment (fertilization level) on SLA?

In a study of loblolly and slash pine conducted in the southeastern United States, significant effects on SLA due to genetic variation between species were observed (Colbert et al. 1990). Interspecific variation does not seem farfetched as different species are significantly varied in genetic composition and are adapted to fill their own respective niche. But what is to be had of *intra*specific variation? Does there exist enough variation within a species to have a significant effect on SLA? Not surprisingly these questions have been already answered, for SLA has been shown to be genetically encoded and significant variability does exists between individuals of a species (Pierce et al. 1994). This led me to hypothesize that genetic variability would have a significant effect on SLA.

Other studies have shown a response in SLA to environmental factors, such as fertilization (Fahey et al. 1998). Some even noting a lower SLA in leaves of nutrient-rich fertilized trees. The availability of resources such as light, water, and nutrients for growth during leaf expansion is an important determinant of SLA. Pierce et al. (1994) found in a study of western U.S. conifer forests that there were no significant differences in canopy-average SLA between fertilized and control plots. This conflicts with the Fahey et al. study, but a notable distinction between the two is that the Fahey et al. study was conducted on deciduous species, while the Pierce et al. study was conducted on coniferous species. Given this information, I hypothesized that I would see a reduction in SLA with increasing nitrogen fertilization levels.

An interaction between genotype and fertilization level seems unlikely, but it is not out of the realm of possibility. Colbert et al. (1990) detected significant effects of treatments × species interactions for leaf area index (LAI), but again this seems unlikely to be true for SLA despite having merit for LAI. My hypothesis for the interaction of genotype and fertilization level was that there would be no significant effect observed.

METHODS

Study Area Description

The experimental site is located near Kulbäcksliden, Sweden (64° 9' 6.5154"N, 19° 35' 9.5994"E) at an elevation of 298 meters. The area is generally flat despite residing on a knoll. Mean monthly temperatures are below freezing for six to eight months out of the year, and summers are short and mild, with long days. Annual precipitation totals are mostly less than 500mm (19.69 in), with a concentration in the summer. Mean annual temperatures are -8.9°C (16.0°F) and 15°C (59.0°F) for January and July, respectfully.

The experimental trees are a part of a larger garden located in a clearcut made in 2007. After the clearcut, the soil was scarified leaving localized depressions. The garden is enclosed within a fence that keeps out larger mammals, such as moose, while allowing smaller animals, such as voles, to gain entrance. The trees were planted in August of 2010, with the fertilizer treatment beginning in 2011.

Treatments

Ten aspen genotypes from the Swedish Aspen (SwAsp) Collection were utilized in this study to establish the genotypic treatment. The trees of the SwAsp collection were collected from 12 different sites in Sweden during the spring of 2003 (Luquez et al. 2007). The genus *Populus* serves as an excellent basis for which to study the effects of genotypic variation due to the extensive research on the subject, in part facilitated by the SwAsp collection.

Environmental factors have a pivotal role in influencing the ways genes are expressed. Specific leaf area (SLA) has also been shown to respond to environmental factors such as fertilization (Fahey et al. 1998). In this study, a gradient of nitrogen conditions was utilized. The three nitrogen fertilization treatments were as follows: control, low, and high treatments with 0, 15, and 150 kilograms per hectare per year of nitrogen (applied in the form of ammonium nitrate) respectfully. The ammonium nitrate was applied in pellet form within a 0.5 meter diameter ring surrounding the base of each treated tree. These treatments were conducted three times per year, at the end of May, June, and July, to meet the aforementioned application rates. These treatments were initiated in 2011 and continue to be conducted for further research.

Sampling Strategy

Not all of the original 300 trees planted in the common garden were suitable for this study. First, 40 trees were lost due to natural mortality. Another two trees were disqualified from the study due to their lack of foliage. The remaining 258 trees were sampled from with 1 to 12 fully expanded, mature leaves being collected depending on the size and vigor of each tree. Scissors were used to avoid induction when removing the leaves at the base of the petiole. The first fully expanded, mature leaves were picked to be representative of the crown of the tree. Upon harvest, the leaves were placed in pre-labeled paper bags and immediately stored in a cooler until brought back to the lab.

The samples were stored overnight in a 5°C cooler closet. The following day the samples were brought into the lab to be scanned for the measurement of leaf area. During this time, samples were stored in coolers until immediately before and after scanning.

Measuring Leaf Area

The set of 1 to 12 leaves from each tree were scanned using a standard, flatbed office scanner at 600 dpi. The raw images were then processed in the following steps:

1. *Make Images Binary:* The raw images were converted into binary form (black and white) using a batch macro in ImageJ. The code for the macro is included in Appendix A.
2. *Measure Area:* Again using a batch macro in ImageJ, the binary images were measured by first setting the scale of the images, then adjusting the threshold to highlight only the black pixels. Lastly, the tool Analyze Particles was used to measure the select pixels. To avoid measuring noise in the images, such as black edges or spots that were not leaves, a minimum area of one square centimeter was chosen. The macro used for this step is included in Appendix B.

Measuring Weight

Following scanning, the leaves were dried in a freeze-drier for 7 days to remove all moisture. Immediately after removal from the freeze-drier, the samples were weighed to the nearest thousandth of a gram. The samples were then place in a -20°C freezer for further use in future studies.

After all the samples had been weighed, the data was combined in a final table containing each trees ID number, the genotype, the fertilization level, and the specific leaf area calculated from the measured leaf area and weight.

RESULTS AND DISCUSSION

Sample Sizes

The sample sizes for each treatment in this study can be seen on the right. An ideal study would have equal sample sizes across each treatment, but due to the inevitability of natural mortality the goal was not met. Thirty trees from each genotype were originally planted in the common garden, but current genotype sample sizes range from 22–30.

One notable trend is that the nitrogen fertilization sample sizes decrease along an increasing fertilization gradient. Originally the trees were randomly, but equally, distributed among the three levels of treatment, but now the sample sizes have a range of 25. Perhaps this is an artifact of the dilemma between growth and defense. As the carbon/nitrogen (C/N) ratio of the soil is decreased by the addition of ammonium nitrate, so is the C/N ratio within the tree. Because of this, C-based secondary metabolism will then decline as growth receives the allocation priority (Herms and Mattson, 1992).

Sample Sizes

		N
N Fertilization	Control	97
	Low	89
	High	72
Genotype	5	30
	18	25
	23	22
	26	25
	50	29
	51	24
	60	29
	65	22
	72	25
	115	27

Levene's Test of Equality of Error Variances

In order to assess the suitability of the data for use in analysis of variance (ANOVA) a Levene's test was conducted. With a significance of 0.021 the data was found to be not suitable, as a significance of 0.05 or greater is required to not reject the null hypothesis of homoscedasity. Because the null hypothesis was forced to be rejected, the data must be transformed in order to perform ANOVA. A transformation of log base 10 was chosen for this data set for its fitness in use with naturally occurring measurements and size data.

A post-transformation Levene's test yielded a significance of 0.085, well above the required 0.05. The null hypothesis was not rejected, and an ANOVA was conducted. The results of the analysis between the dependent variable (SLA) and the treatments are listed in the table below.

Tests of Between-Subjects Effects

Source	df	F	Sig.
N Fertilization	2	56.702	.000
Genotype	9	10.847	.000
N Fertilization x Genotype	18	1.407	.129

In ANOVA a significance of less than 0.05 is considered significant. The significance value represents the probability that the data could have been the result of random chance alone. With values of zero for both treatments exclusively, it can be concluded that nitrogen fertilization and genotypic variation each have a significant effect on SLA. Conversely, the effects both treatments combined are not likely to be significant.

Post Hoc Tests

In order to more precisely determine where the significance occurs within each treatment, several post hoc tests were conducted. The three post hoc tests were: Boferroni, Gabriel, and Hochberg GT2. All of the tests produced the same pattern of significance despite the individual values varying slightly. Hochberg's GT2 test was ultimately chosen for this analysis as it is the most suitable when there is high variance among sample sizes (Field, 2009). The results of Hochberg's test are listed in the table below.

Hochberg GT2 Post-Hoc Analysis – Level of Significance

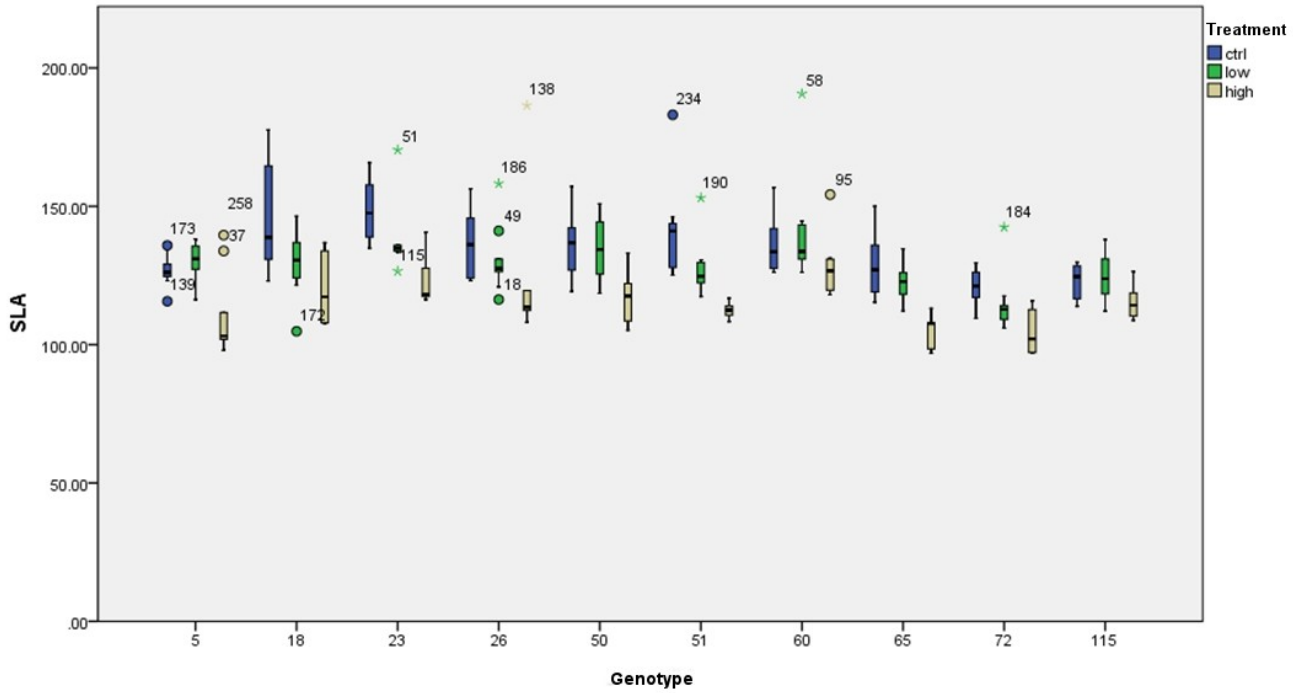
Genotype	**5**								
18	0.016	**18**							
23	0.000	0.999	**23**						
26	0.163	1.000	0.801	**26**					
50	0.414	1.000	0.335	1.000	**50**				
51	0.495	1.000	0.453	1.000	1.000	**51**			
60	0.001	1.000	1.000	1.000	0.969	0.989	**60**		
65	1.000	0.012	0.000	0.114	0.295	0.356	0.001	**65**	
72	0.336	0.000	0.000	0.000	0.000	0.000	0.000	0.807	**72**
115	1.000	0.004	0.000	0.048	0.146	0.195	0.000	1.000	0.806

N Fertilization	**Control**	
Low	0.021	**Low**
High	0.000	0.000

No Significant Difference

Significant Difference

Highlighted values represent a significant difference (≤ 0.05) in the comparison of two treatments. Significant differences can be seen between genotypes, but not all are significant. Conversely, all differences between nitrogen treatments are significant. These observations can also be seen in the graph below.



SUMMARY AND CONCLUSIONS

Genetic variation within a species (between genotypes) does appear to have significant effect on SLAs as expected. Not all of the genotypes in this study varied significantly from each other, but almost a third did which is enough to confirm my hypothesis. There also appears to be significant variation in SLA along a gradient of nitrogen conditions. Looking at the mean SLA for each nitrogen treatment in the table below, a decreasing trend can be easily seen.

Means for Nitrogen Treatments

N Treatment	Mean SLA (cm^2/g)
Control	133.690
Low	129.003
High	115.744

*Means transformed back for interpretation

Again the results met my expectations showing that SLA decreases with increasing nitrogen conditions. The only treatment not found to have a significant effect on SLA was the combined interaction of nitrogen fertilization × genotype. But again this was expected because although each has a significant effect on their own, it did not seem likely that combined they would have a additional effect.

APPENDIX A.

```
// This macro converts all the files in a folder to binary. The binary

macro "Batch Convert to Binary" {
requires("1.33s");
dir1 = getDirectory("Choose Source Directory ");
dir2 = getDirectory("Choose Destination Directory");
list = getFileList(dir1);
setBatchMode(true);
for (i=0; i<list.length; i++) {
path = dir1+list[i];
open(path);
run("8-bit");
setAutoThreshold();
run("Apply LUT");
dotIndex = lastIndexOf(path, ".");
if (dotIndex!=-1)
path = substring(path, 0, dotIndex); // remove extension
save(dir2+list[i]);
close();
}
}
```

APPENDIX B.

```
// This macro measures the area of leaves aready in binary form with with an empty white background

macro "Batch Measure Area" {

requires("1.33s");
dir1 = getDirectory("Choose Source Directory ");
dir2 = getDirectory("Choose Destination Directory");
list = getFileList(dir1);
setBatchMode(true);
for (j=0; j<list.length; j++) {

path = dir1+list[j];
open(path);
run("Set Scale...", "distance=600 known=5.1 pixel=1 unit=cm global");

setAutoThreshold("Default");
//run("Threshold...");
setOption("BlackBackground", false);
run("Convert to Mask");
run("Analyze Particles...", "size=1-Infinity circularity=0.00-1.00 show=Nothing summarize");
}

//saveAs("Results", dir2+"Results.xls");
saveAs("Text", dir2+"Summary.xls");
close();
}
```

LITERATURE CITED

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