Sampling Leaves of Young Potato (*Solanum tuberosum*) Plants for Glycoalkaloid Analysis

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Earlier attempts to measure potato (*Solanum tuberosum*) leaf glycoalkaloids indicated variability among similar plants, suggesting that a single small sampling of a young plant might not be a reliable measure of composition. It was also suggested that freeze-dried leaf samples might be less variable than fresh ones. In the present work, variability was minimized by comparing single leaves from the same stem position of each plant. Comparisons involving other leaves indicated that the glycoalkaloid content was not constant with respect to either time or position on the stem. In addition, the possible influence of differences in growing conditions at different times suggests that repeated plantings should include a known variety as a control to which other plants are compared. Variability was reduced by calculating glycoalkaloid concentrations on a dry weight rather than fresh weight basis. The method of drying the samples, however, had no influence on the variability of data. These considerations should be generally applicable to the sampling of replicate leaves of any plant for analysis of any components.

Keywords: *Glycoalkaloid; leaf sampling; potato; α-solanine; α-chaconine; freeze-drying; breeding*

INTRODUCTION

Need for Reliable Glycoalkaloid Determinations. Breeding programs intended to introduce desirable growing or culinary factors may introduce undesired characteristics also. Potatoes (*Solanum tuberosum*) may produce undesirably high or low levels of glycoalkaloids, including, but not limited to, α-solanine and α-chaconine (Friedman and McDonald, 1997). Other components may be of interest in other crops. To utilize growing space most efficiently, early sampling and measurement of leaf components in seedlings would increase the efficiency of the program by allowing early discarding of those that do not meet the desired parameters. Minimizing the size of the removed leaf sample and allowing as much photosynthetic area as possible to remain should allow growth of the retained plants to continue with little disturbance. A reliable method of early leaf analysis could then be used in studies of the correlation of foliar and tuber alkaloid content.

Sources of Variation in Potato Glycoalkaloid Determination. The measurement of glycoalkaloids in potatoes has been addressed in several earlier publications from this and other laboratories. In the analysis of tubers, there was little variation in concentration between replicate tubers (Dao and Friedman, 1994). Papathanasiou et al. (1999) found that the smallest tubers on a plant had higher concentrations of glycoalkaloids than the other tubers, but the variation was consistent for two cultivars in two successive years. Results were similarly consistent when a large number of leaflets of each plant were freeze-dried, ground, and mixed prior to analysis (Dao and Friedman, 1994). However, the glycoalkaloid content of individual leaflets selected at random and not freeze-dried was found to be quite variable (Dao and Friedman, 1996). In that paper, it was suggested that freeze-drying reduced the variability, in comparison to direct analysis of the fresh leaflets for which the standard error of the mean was ~4 times as great.

Evaluation of Young Plants. The ability to determine glycoalkaloid content from a single leaf would be valuable in breeding programs because of its minimal disturbance of continued growth. Analysis of petioles has been a recommended technique for assessing the mineral nutrient status of plants for >50 years (Ulrich, 1952), but the low concentration of nutrients there requires collection of a number of units, making it inappropriate for analysis of individual small plants.

Criteria for Sample Selection. One study of the nutrient contents of the upper six petioles of potato plants has demonstrated significant differences among them (Westermann et al., 1994). Other studies showed the influence of leaf age on rates of photosynthesis in potato leaves (Vos and Oyarzun, 1987), on photosynthesis and mineral nutrient composition of soybean leaves (Brown and Bethlenfalvay, 1987), on the rapid rise and gradual decline of hemagglutination activity in potato leaf extracts (Wierzba-Arabska and Morawiecka, 1987), and on various aspects of leaf metabolism in cotton (Wells, 1989). Thus, it is reasonable to assume that the concentration of glycoalkaloids in potato leaves might also change as leaves age. Although there are physiological differences among leaves of a plant, proper sample selection should prevent those differences from introducing any unrecognized variables into an experiment.

Influence of Sample Handling. The generally accepted practice of determining glycoalkaloid concentrations of potatoes on a fresh weight basis is an additional source of variability. Plant moisture level at any time is a function of soil moisture level, atmospheric relative humidity, and air movement around the plant, superimposed on the normal diurnal cycle (Ulrich, 1952). Furthermore, the normal transpiration of water

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from a leaf does not cease instantly upon removal of the leaf from its plant. Thus, even an accurately determined weight of a leaf as it was attached to the plant, preferably accomplished by enclosing it in a vaporproof wrapping before cutting it from the stem, will still include an uncertain amount of water. On the other hand, if the water content of all tissue samples is reduced to a common very low value by drying in an oven at 70−80 °C for a day, weights representing tissue size and composition can then be measured within ∼1%. Dried leaves should, of course, be weighed or enclosed promptly after a brief cooling period to avoid absorption of water from the atmosphere. Such water uptake is much slower than the loss of water from the freshly cut leaves. However, if left exposed for a day, dried leaf tissue can absorb enough water from a moist atmosphere to increase its weight by 10−15% (M. S. Brown, unpublished data).

Evaluation of Sources of Variability. This study was designed to evaluate certain factors affecting variability in comparative measurements of the glycoalkaloid content of leaves, including both their position on the stem and their age from emergence, as well as the handling of samples after removal from the plant. The physiological functions responsible for these observed differences were not investigated. Development of a sampling method based on this information should permit reliable comparative measurements using a single leaf from each plant, thereby leaving most of the leaf area to support future growth of those seedlings that are to be retained.

MATERIALS AND METHODS

Experimental Design. Using data from the earlier leaf analysis experiment (Dao and Friedman, 1996), confidence interval was plotted versus number of replications. Although six replications yielded a 95% confidence interval of 5.5% of the mean, increasing the number of replications by 50% (to nine replications) reduced the confidence interval by only an additional 1.2%. Therefore, an experiment was designed with six replications each of three groups of plants, with each plant to be sampled only once. The single sampling time for each individual plant avoided the possible consequences of early removal of a leaf on the subsequent glycoalkaloid synthesis and/or accumulation in that plant. In sampling A, leaflets from one leaf of each plant were freeze-dried, whereas those above and below were oven-dried. Leaf samples from all other plants were oven-dried. Figure 1, a schematic representation of the plants at the end of the experiment, shows the locations and times of sampling. Numbered horizontal lines indicate the positions of leaves on the stem, and the letters indicate which leaves were included in each sampling. Data from analysis of the A leaves were intended to answer the primary questions about position of the sampled leaf on the stem and the method of drying the sample. Other sets of six plants each were sampled at two earlier times (B, 17 days; C, 32 days) than the A plants only to see whether the glycoalkaloid composition remained constant over time. The reasons for possible differences were not studied, and in plants grown under different conditions, the glycoalkaloid levels might or might not change similarly with time. There was no intent to determine a detailed chronology of those changes, if any.

Plants. Organically grown Russet potatoes not treated with sprout inhibitor were purchased from a local grocery store. Tubers were cut in fourths, dried, and planted in 24 4-L pots in a greenhouse. The greenhouse temperature range was from 27 °C during the day to 17 °C at night. After the sprouts emerged, plants were thinned to one shoot per pot, and after an additional week of growth, 18 similar plants were selected for the experiment.

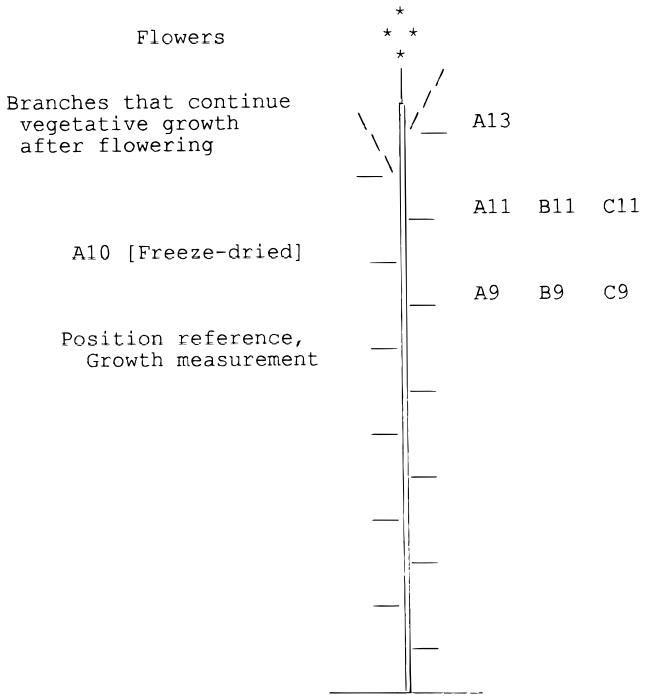

Figure 1. Schematic representation of potato plant showing leaf positions and sampling times. Horizontal lines indicate positions of leaves on the stem (leaf number). Letters denote sampling days (days after shoot emergence): A = 37; B = 17; C = 32. Each plant was sampled only one time, although more than one leaf was taken from each plant (i.e., four A leaves, two B leaves, or two C leaves). No leaf samples were taken from unlettered positions. Leaves from position 10 were freeze-dried; those from positions 9, 11, and 13 were oven-dried.

The first 8 (± 2) leaves on these plants did not have the typical 7-leaflet compound configuration of all later leaves. On the chance that other aspects of metabolism were also abnormal in these early leaves, sampling for glycoalkaloid analysis was restricted to those leaves of normal configuration below the nodes at which flower buds first developed. This differed from the procedure of the earlier study (Dao and Friedman, 1996), in which only the first five or six leaves were analyzed. Ten days after shoot emergence, each shoot was tagged at what appeared to be the uppermost fully expanded leaf. Daily measurement of the midrib lengths of the tagged leaves showed that they continued to grow as much as 50% during the next three to five days. This initial misjudgment of size as "fully expanded" was due to the fact that, during early growth, each leaf becomes larger than the one preceding it. In this case, the evaluation of "fully expanded" was based on the leaf below the one selected, which the newer leaf had equaled or exceeded. Nevertheless, it provided a permanent reference point on the stem for locating subsequent leaves to be sampled, in case one or more of the lowest leaves senesced and dropped from the stem. Lateral shoots were removed from the lower leaf axils as soon as they emerged, to ensure that the plants remained as uniform as possible. With the onset of flowering, strong vegetative shoots developed from the two leaf axils just below the first flowers, and these were allowed to remain.

At the appropriate sampling time, the rachis of the leaf (equivalent to the midrib of a simple leaf; sometimes included, for simplicity, in the term "petiole") was cut as close as possible to the three terminal leaflets, separating them as a unit from the plant. The next two opposing leaflets were also cut from the rachis. These five leaflets comprised the leaf tissue sample from that plant. In one of the groups, leaflets were weighed immediately after removal from the plant (fresh weight). Leaflets to be freeze-dried were placed on dry ice (solid carbon dioxide) immediately after being weighed and, when frozen, were placed in paper envelopes for storage in a freezer. The

Table 1. Glycoalkaloid (GA) Content of Potato Leaves

		concn (g/kg of dry wt) at leaf position			
day[a]	GA	9	10	11	13
A	α-solanine	2.23 y	3.03 z	1.84 y	1.14 x
	α-chaconine	3.54 y	4.50 z	3.43 y	2.38 x
B	α-solanine	3.37	*b*	1.59	*b*
	α-chaconine	5.64	*b*	3.37	*b*
C	α-solanine	1.56	*b*	1.73	*b*
	α-chaconine	3.31	*b*	3.45	*b*

[a] Day of sampling (days after emergence): A = 37, B = 17, C = 32. Day A: For each glycoalkaloid, values followed by the same letter are not significantly different at $p = 0.05$ (ANOVA, Bonferroni *T* test). Days B and C, for each alkaloid, differences are not significant. [b] Leaf position not sampled on these days.

Table 2. Variability of Glycoalkaloid (GA) Analyses of Potato Leaves after Freeze-Drying or Oven-Drying[a]

leaf position	drying	GA	SD	CV
10	freeze	α-solanine	0.54	17.7
		α-chaconine	0.73	16.3
9	oven	α-solanine	0.41	18.6
		α-chaconine	0.66	18.7

[a] Each figure is derived from analysis of six replications. SD, standard deviation; CV, coefficient of variation.

frozen leaflets were freeze-dried within the following week. Other leaflets in paper envelopes were placed into an oven at 70 °C within 1 h after removal from the plants. The dry leaflets were weighed and then ground in a small coffee mill (Braun KSM model 4041).

Glycoalkaloid Analysis. Accurately weighed portions (~50 mg) of the oven-dried leaf tissue were extracted by stirring for 2 h with 40 mL of 2% acetic acid. Each extract was filtered, and the filtrate was made basic with NH_4OH (solution color change serves as the indicator). This basic solution was extracted twice with 20 mL of water-saturated *n*-butanol. The butanol layers were combined and evaporated to dryness. The residue was redissolved in 2 mL of methanol and diluted with 2 mL of acetonitrile and 1 mL of water. Aliquots of this solution were injected directly into the chromatograph for glycoalkaloid analysis. Samples were extracted in duplicate, with triplicate HPLC analyses of each solution.

A Beckman (Fullerton, CA) model 334 liquid chromatograph with a Dionex (Sunnyvale, CA) 4400 integrator and a Beckman 165 UV−visible variable wavelength detector was used. The column was 3.9 × 300 mm with Resolve C_{18} packing (Waters, Milford, MA). Eluent was 100 mM monobasic ammonium phosphate in 35% acetonitrile, adjusted to pH 3.5 with phosphoric acid. Flow rate was 1 mL/min, and detection was at 200 nm.

RESULTS

The influence of leaf position on glycoalkaloid concentration on day 37 (from shoot emergence) is shown in Table 1, day A. These data show decreasing glycoalkaloid concentrations from leaf 10 to 13, along with lower values in the oldest leaf sampled, from position 9. Compositions of leaves 9 and 11 at the earlier sampling dates (days B and C) were sufficiently variable that differences were not significant.

In Table 2, the variability of α-solanine and α-chaconine concentrations in the freeze-dried leaves (leaf position 10) are compared with those of the oven-dried leaves immediately below them on the stem (leaf position 9). Differences in the standard deviations are a reflection of the differences in mean concentrations of the two glycoalkaloids in the leaves. The coefficients of variation, which correct for these concentration differences, are essentially the same for α-solanine and α-chaconine.

Table 3. Ranking of Fresh Weight (FW) and Dry Weight (DW) of Six Replicate Leaf Samples, from Lightest (1) to Heaviest (6), plus Ratio of Heaviest to Lightest (H/L) for Each Leaf Number (Sampling Day A)

leaf position		rank						H/L
		repl 1	repl 2	repl 3	repl 4	repl 5	repl 6	
9	FW	6	1	5	2	4	3	1.44
	DW	6	4	3	5	2	1	1.22
10	FW	2	5	6	3	4	1	1.65
	DW	2	5	6	4	3	1	1.61
11	FW	2	1	6	4	5	3	1.44
	DW	1	2	4	6	5	3	1.63
13	FW	1	3	4	5	2	6	1.71
	DW	1	3	4	5	2	6	1.92

The variability introduced inadvertently by the use of leaf fresh weight as the basis for glycoalkaloid concentration calculations is indicated by the data of Table 3. In each row, the leaf weights are ranked from lightest (1) to heaviest (6). Changes in rank of the six replicates of each leaf number, from fresh weight (FW) to dry weight (DW), indicate that the samples lost different amounts of water during drying. In only one of the four sets of leaf samples was the order of heaviest to lightest replicates maintained through the drying process, but even there the ratio of heaviest to lightest increased 12%. If the percentage of water in all of the leaves of a set had been the same, neither the rank orders nor the ratios of heaviest to lightest would have been changed by drying.

The water contents of the fresh leaves were 87−92% [(FW − DW)/FW], a relatively small range. Solids, on the other hand, constituted from 8 to 12% (DW/FW) of the fresh weight of the leaf tissue, a range of 1−1.5.

DISCUSSION

Analyses of a number of leaves sampled on a single day show that the glycoalkaloid content increased with leaf maturity and then declined with further age.

The coefficients of variation for α-solanine and α-chaconine contents were not significantly different between oven-dried and freeze-dried leaf samples. Thus, with uniform sampling, the method of drying the leaves does not affect the variation among individual plants in a group sampled at a single time. The availability of a new ELISA kit (Friedman et al., 1998) for potato glycoalkaloids should facilitate evaluation of large numbers of leaf and tuber samples.

The water content figures seem to be less variable than the dry weights of the same samples, but that is an artifact of the high water content of most plant tissues. Thus, the basis for measurement of the concentration of a particular component should be related to the amount of solids, represented by dry weight, without the uncertain variability of the water content of the tissues. This is particularly true in the case of leaves, which, as mentioned earlier, are subject to both cyclic changes while on the intact plant and continued water loss after removal from the plant.

From these results it is concluded that the variability among individual fresh leaves observed earlier (Dao and Friedman, 1996), with standard errors ~4 times those of freeze-dried leaves, was probably due to the random positions from which individual leaflet samples were selected, further complicated by the variations in water

content, and not to the omission of the freeze-drying step to which the remainder of each group of samples was subjected. In their study, the freeze-dried tissue was composed of leaflets from a number of leaves, which would have tended to average the differences due to individual leaf age and water content.

In conclusion, both time of sampling and the stem position from which the leaf sample is taken can influence the observed glycoalkaloid concentrations. Thus, it is important to make comparisons at a specific time, sampling the same leaf from each plant. Differences in growing conditions at different times or locations undoubtedly affect these data also. In an ongoing program involving repeated plantings and analyses, plants of one or more control varieties should always be grown. Differences between plantings should be reported only as differences from their respective control plants. On the other hand, the variability among replicates was not reduced by freeze-drying. Leaf samples should probably be oven-dried "promptly," although the effect of delay between sampling and drying was not investigated in the experiments reported here.

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