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FROZEN VERSUS NON-FROZEN SAMPLE PREPARATION FOR PLANT TISSUE PHOSPHORUS ANALYSIS

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ABSTRACT

Maize (Zea mays L.) and wheat (Triticum aestivum L.) plant tissue was collected from phosphorus fertility experiments at two locations in 1985. Two samples were collected from each experimental unit and randomly either frozen immediately following collection or stored and dried normally prior to being analyzed for total P (HNO_3-$HClO_4$ acid digest) and inorganic P (CH_3COOH extract). Corn was sampled at the eight and twelve leaf stages and wheat at Feekes stage five. Freezing was accomplished using liquid N (wheat-Feekes stage five) and dry ice (corn-eight and twelve leaf stages). Inorganic P was found to be greater when frozen at the twelve leaf stage versus the

conventional non freezing procedure. Alternatively, the opposite was found for wheat at Feekes stage five. The freezing procedure was found to increase the total P concentration for both corn at the twelve leaf stage and wheat at Feekes stage five. Losses of CO_2 were expected in the conventional non-frozen samples due to an induced leaf respiration (eight hours from the time samples were collected to the time they entered the drying oven) which should have reduced dry weight and subsequently increased concentration. Because this was not found and since all other procedures employed were identical other than freezing, it is expected that P volatilization losses must have occurred in the non-frozen samples for corn at the twelve leaf stage and wheat at Feekes stage five.

INTRODUCTION

Accurate determination of a given nutrient at the time of sampling is essential in plant nutrient uptake studies. Errors associated with plant sample analysis have been attributed to soil contamination[10,17], drying temperatures[5,7,8,10,15,17], random laboratory contamination[10,17], grinding procedures[10,17], plant sample decay prior to analysis[6,7] and initial temperature storage prior to drying[15]. The effect of dry weight losses which result in increased nutrient concentration when expressed on a dry weight basis has also been considered[15]. Other work relative to temperatures has indicated that organic P compounds may be hydrolyzed upon heating thus affecting PO_4-P extraction by method of preparation[8]. High atmospheric concentrations of CO_2 have been shown to cause stomata to close thus inhibiting leaf respiration[2]. Considering all of these factors, it must be assumed that final prediction of concentration is a function of the cumulative errors in each preparatory phase[17]. Bias and or differing accuracy introduced by method of preparation for corn or wheat plant tissue could alter predicted total P, inorganic P

and final uptake values. This could then lead to altered correlation and or interpretation with other calibration parameters such as soil test levels.

The objective of this experiment was to determine the effect of freezing plant tissue immediately following collection on the concentrations of total P and inorganic P and calculated uptake versus that of a conventional drying procedure.

MATERIALS AND METHODS

Wheat (*Triticum aestivum* L.) and corn (*Zea mays* L.) plant tissue was collected from phosphorus fertility experiments at two locations in 1985 and subjected to different freeze procedures prior to analysis. Plant samples were either frozen immediately after collection or stored non-frozen prior to being analyzed for total P and inorganic P. Wheat was sampled at Feekes stage 5 (Croy L.I., 1959. M.S. Thesis. Oklahoma State University) at one location near Imperial, Nebraska. Two samples of thirty plants (roots removed after sampling) were taken from each plot. After collection, the two samples from each plot were randomly either frozen or processed conventionally (stored in paper bags prior to drying in the forced air oven). Initial freezing took place by wrapping samples in aluminum foil and dipping into liquid N (-196C) for one minute. Frozen samples were allowed to thaw and then re-frozen in a conventional freezer at -24C. Non-frozen samples were dried in an air forced oven at 70C eight hours after collection. Frozen samples were dried in this same manner following the second freezing.

Corn was sampled at the eight and twelve leaf stages from a P-fertility experiment near Loup City, Nebraska. Eight whole plants per plot (roots removed) and 10 of the fully most developed leaves were collected at the eight and twelve leaf stages respectively. Two samples were taken from each plot, one frozen using dry ice in 'Coleman' coolers (approximately -78C)

and the other stored at ambient temperature (29C) in paper bags prior to drying. At the twelve leaf stage, the two plant samples were randomly clipped to obtain the same initial wet weights (+/- 0.5g) before either freezing or not freezing. Frozen samples from the corn experiment were also re-frozen at -24C in a conventional freezer once thawed. After the second freezing, plants were allowed to thaw and then dried in a forced air oven at 70C. Non-frozen samples were dried in the same manner after returning from the field (eight hour period from the time samples were collected to the time they were entered in the drying oven - ambient temperature for the non-frozen samples prior to drying was approximately 29C). Initial wet and dry weights were not taken for wheat at Feekes stage five or for corn at the eight leaf stage.

Once dry, both frozen and non-frozen samples were ground to pass a 40-mesh screen, and a 0.5g sample was analyzed for total P by HNO_3-$HClO_4$ acid digestion excluding H_2SO_4 acid and developing color by the vanadomolybdate procedure[1,4]. All samples were also analyzed for PO_4-P (inorganic P) on 0.2g samples by shaking with 50 ml of 0.348M (2 percent) CH_3COOH acid at 25C for 30 minutes. Phosphate-phosphorus concentration was determined after filtration through Whatman No.2 paper using the phosphomolybdate colorimetric procedure of Murphy and Riley[11].

Uptake of P (total P and inorganic P) in corn at the twelve leaf stage was determined by multiplying concentration as percent times the dry weight. These values are reported in grams.

RESULTS AND DISCUSSION

Total P in frozen samples of wheat taken at Feekes stage five was significantly higher than in non-frozen samples (Table 1). This was also noted for corn at the twelve leaf stage, however, no differences in total P concentration at the eight leaf stage were detected between the frozen and non-frozen

TABLE 1

Means for main effect variables when frozen and non-frozen and associated differences from the analysis of variance model.

Variable	Frozen	Non-frozen	PR>F	CV
12L, Wet Weight, g	134.37	133.06	NS	22
12L, Dry Weight, g	28.37	28.06	NS	12
12L, Total P, ug g^{-1}	2288.2	2058.2	*	15
12L, Inorganic P, ug g^{-1}	1463.3	1023.6	**	18
12L, % Inorganic P	64.2	49.2	**	11
12L, Total P Uptake, g	0.0657	0.0586	*	21
12L, Inorganic P Uptake, g	0.0421	0.0292	**	23
8L, Total P, ug g^{-1}	4137.2	4583.3	NS	11
8L, Inorganic P, ug g^{-1}	2113.2	2187.5	NS	12
8L, % Inorganic P	51.4	47.7	NS	9
F5, Total P, ug g^{-1}	2645.8	2058.3	**	18
F5, Inorganic P, ug g^{-1}	632.9	781.8	*	29
F5, % Inorganic P	23.1	37.9	**	14

*,** - significant at 0.05 and 0.01 probability levels respectively. NS- not significant. 8L, 12L- eight and twelve leaf stages, corn, respectively. F5 -Feekes stage five, wheat. CV -coefficient of variation, percent. PR F-probability of a greater F statistic.

samples. Relative increases in total P concentration should have been observed in the non-frozen samples due to expected losses of CO_2 during an induced respiration immediately following sampling[15]. The actual wounding or disruption of plant tissue which was evident for all samples taken (both frozen and non-frozen - various folds of the leaves prior to storage in the paper bags employed) has been reported to greatly stimulate respiration[2]. Since concentration is a function of actual dry matter used and since P volatilization losses during

respiration were not anticipated, it is difficult to explain these results. However, it is hypothesized that either P volatilization losses were significant or that respiration substrates, adenosine diphosphate and inorganic phosphate were abnormally consumed in the non-frozen samples. Although not significant, total P in non-frozen samples at the eight leaf stage were slightly larger than when frozen. Previous work has indicated that respiration rates are much higher in younger plant tissue[9]. Since whole plants were sampled intact at the eight leaf stage (versus fully most developed leaves - 12 leaf stage), actual respiration rates may well have been greater for this sampling which aid in explaining these results.

By use of the acetic acid extract, significant increases in inorganic P when plant tissue was frozen were found for corn at the twelve leaf stage. However, this was not observed for corn at the eight leaf stage or for wheat at Feekes stage five. By use of the freezing methods employed, it was expected that the vacuole would be ruptured which is a known storage house for orthophosphate anions thus increasing the dilute acid extractable inorganic P forms[3,13,14,16]. The calculated heat of freezing was not sufficient to break carbon-carbon bonds thus releasing organic P forms[18]. However, rupturing of cell walls should have taken place via the expansion of water upon freezing. While this mechanism was believed to be responsible for the increased inorganic P in the frozen twelve leaf stage samples, contrasting results in the wheat data are more difficult to explain.

The percent of total P as inorganic P was significantly higher for corn at the twelve leaf stage when frozen compared to non-frozen (Table 1). No differences were observed for the percent of total P as inorganic P at the eight leaf stage. Alternatively, the percent of total P as inorganic P at Feekes stage five was greater when non-frozen versus frozen. It is

interesting to note that the percent of total P as inorganic P was much higher for the frozen samples at the twelve leaf stage versus that found at the eight leaf stage which is consistent with previous work documenting higher inorganic P levels in older tissue[9]. Alternatively, when non-frozen, the percent of total P as inorganic P was virtually the same at the twelve and eight leaf stages (Table 1). These data suggest a dynamic change in procedure on the amount of acetic acid extractable P as well as differences in growth stage and crop sampled.

Total P and inorganic P uptake values determined at the twelve leaf stage were higher when frozen compared to non-frozen (Table 1). The expected loss in dry weight (CO_2 evolution in non-frozen samples) and a subsequent increase in concentration should have balanced final predictions of uptake for total P and inorganic P when non-frozen versus frozen. However, dry and wet weights were not significantly different for non-frozen and frozen samples which further suggests that phosphorus volatilization losses may have been present in the non-frozen samples. While only total P and inorganic P analysis was run on these samples, it is of some concern as to how predicted uptake values for other elements will be affected by freezing versus non freezing.

Correlation, intercepts and slopes for total P - inorganic P regression equations when frozen and non-frozen are shown in Figure 1. Previous work has shown a high correlation between the inorganic P and total P procedures[12]. Because of this, organic P regression equations were of interest based on sample preparation. For both corn samples, intercepts were not significantly different when frozen versus non-frozen (Table 2). However, intercepts were found to be different for wheat at Feekes stage five. For corn this indicates that either procedure was within accurate ranges of estimating total P when inorganic P equals zero. This also indicates that no bias was

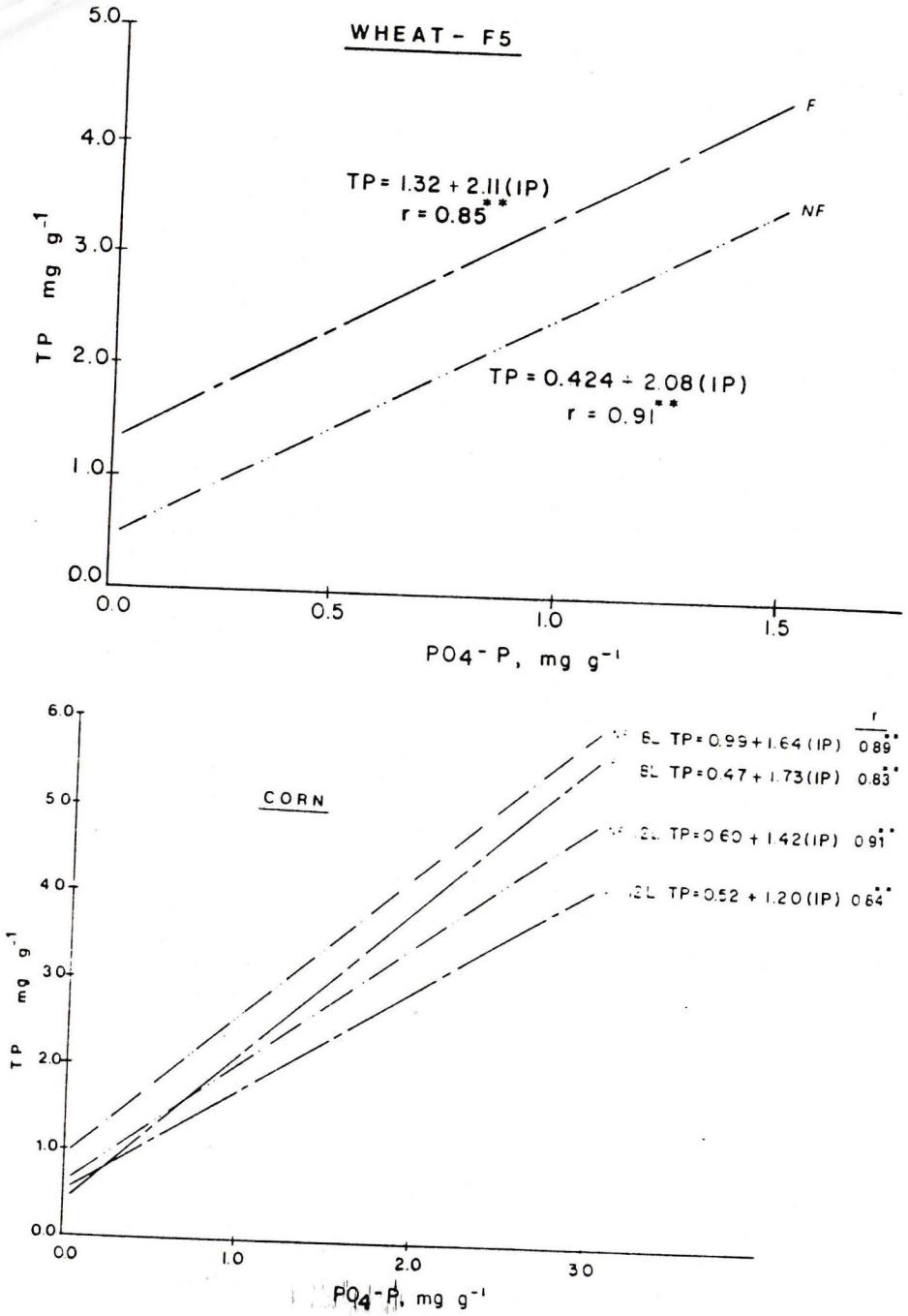

FIG 1. Regression analysis of total phosphorus versus phosphate-phosphorus when plant tissue was frozen and non-frozen for corn at the eight and twelve leaf stages and for wheat at Feekes stage five. NF- non-frozen, F-frozen.

TABLE 2

Differences in slopes and intercepts for total P - inorganic P regression equations for frozen versus non-frozen corn and wheat samples.

Stage	F-calc ----- F MSE	PR>F -----	t-calc -- t slope	PR>F ---	t-calc - t intercept-	PR>F
Eight leaf stage, corn	2.59	**	0.54	NS	1.49	NS
Twelve leaf stage, corn	1.96	**	1.87	@	0.50	NS
Feekes stage five, corn	1.87	*	0.15	NS	5.67	**

@,*,**- significant at 0.10, 0.05, and 0.01 probability levels respectively. NS-not significant. PR>F-probability of a greater F statistic.

introduced into the estimate of intercepts based on sample preparation. For wheat, the opposite of this was found. The non-frozen slope at the twelve leaf stage was significantly greater than when frozen. This slope difference recorded at the twelve leaf stage leads to differences in accuracy when estimating total P from inorganic P derived data. By freezing the plant tissue, errors associated with total P prediction should be decreased due to the procedural error introduced when CO_2 losses exist. Correlation coefficients were somewhat improved when non-frozen versus frozen which possibly suggests that sample preparation errors in this experiment were greater when frozen (Figure 1).

No significant differences were recorded in slopes at either the eight leaf stage or Feekes stage five for frozen versus non-frozen estimates.

TABLE 3

Analysis of variance for main effect variables at the eight and twelve leaf stage samples for corn, from a method-source-rate experiment, Loup City, NE, 1985.

Source of Variation	df	12L Total P Uptake	12L Inorganic Inorganic P Uptake	12L Percent Moisture	8L Total P	8L Inorganic P
		PR > F				
Total	143					
Replication	2	**	*	NS	NS	NS
Sample	1	*	**	NS	NS	NS
Rep*Sample (error a)	2					
Method (M)	3	**	**	**	**	**
Source (S)	2	*	*	*	@	*
Rate (R)	1	**	**	*	**	**
M*S	6	*	**	NS	NS	*
M*R	3	NS	NS	NS	NS	NS
S*R	2	NS	NS	@	*	@
M*S*R	6	*	@	NS	NS	NS
Sample*M	3	NS	NS	NS	NS	NS
Sample*S	2	NS	NS	NS	NS	NS
Sample*R	1	NS	NS	*	NS	NS
Sample*M*S	6	NS	NS	NS	NS	NS
Sample*M*R	3	NS	NS	NS	NS	NS
Sample*S*R	2	NS	NS	NS	NS	NS
Sample*M*S*R	6	NS	NS	NS	NS	NS
Error	92					

@,*,**-significant at 0.10, 0.05, and 0.01 probability levels respectively.
df -degrees of freedom.

TABLE 4

Analysis of variance for main effect variables on wheat tissue samples taken at Feekes stage five from a method-rate experiment, Imperial, NE, 1985.

Source of Variation	df	Total Phosphorus	Inorganic Phosphorus
		PR > F	
Total	89		
Replication	2	**	**
Sample	1	**	*
Rep*Sample (error a)	2		
Method	4	**	*
Rate	2	*	NS
Method*Rate	8	*	*
Sample*Method	4	NS	NS
Sample*Rate	2	NS	NS
Sample*Method*Rate	8	NS	NS
Error	56		

*,**-significant at 0.05 and 0.01 probability levels respectively.
df-degrees of freedom.

Main effect models are listed in Tables 3 and 4. On only one occasion was there a sample (method of preparation) by main effect variable interaction. This suggests that the experimental interpretation of fertilizer application methods, sources and rates employed in these field trials, was not affected by the method of sample preparation (freezing versus non freezing). The exception to this was found for percent moisture at the twelve leaf stage, where frozen samples showed no differences with rates, while moisture levels were higher for the higher P rate (18 vs 9 kg ha-1) when non-frozen. This could indicate lower respiration rates, less CO_2 evolution, decreased dry weight losses and subsequent lower moisture levels for non-

frozen samples at the lower P rate. Alternatively, the field observation of increased vegetative growth at the high P rate was expected to increase moisture levels. Unfortunately, it is difficult to explain why moisture levels were the same when frozen for both P rates. Dry weights must have been correspondingly higher at the high P rate when frozen to account for the lack of differences in percent moisture.

Percent Moisture, Twelve Leaf Stage, Corn

	P rate, kg ha-1	
	9	18
Frozen	78.81	78.84
Non-frozen	78.48	79.16

Differences in freezing procedures will probably need further investigation since the freezing points of the two methods (wheat-liquid N, corn-dry ice) differed substantially. The need for having samples at the same initial wet weight before freezing or not freezing is of considerable importance when final values of uptake are to be determined. Unfortunately, samples were not prepared in this manner for wheat at Feekes stage five or for corn at the eight leaf stage.

CONCLUSIONS

The freezing methods used were found to increase total P in tissue samples taken for corn at the twelve leaf stage and wheat at Feekes stage five versus samples not frozen immediately following collection. While CO_2 losses were expected in the non-frozen samples which would have subsequently decreased dry weight and increased concentration, it is hypothesized that P volatilization losses may in fact be present when plant tissue is not frozen immediately following collection or when long time periods exist prior to oven drying conventionally taken samples.

Freezing was expected to rupture the vacuole which is a known storage house for orthophosphate anions, therefore increasing dilute acid extractable inorganic P forms. While this was in fact observed for corn at the twelve leaf stage, wheat data at Feekes stage five did not parallel this finding. Equations for the prediction of total P using inorganic P were significantly altered by method of preparation, conceivably changing correlation with yield and or soil test data. The method of preparation (frozen versus non-frozen) did not affect rank or order of the other main effect variables included in these experiments therefore indicating that freezing would not affect interpretation of the P tissue analysis. Differences in freezing methods used in this experiment (dry ice and liquid N) were not properly evaluated and will need further investigation.

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