SEED UPGRADE IN *ALNUS TENUIFOLIA*[4]

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ABSTRACT

Little is known about the propagation of thinleaf alder (*Alnus tenuifolia*). This species, native to New Mexico, has potential applications in rehabilitation of disturbed lands, riparian restoration and possibly landscaping. An efficient and economical method for propagation is needed. Thinleaf alder produces prolific amounts of small seed with typically low viability. These characteristics make propagation by seed problematic. Problems encountered with seed propagation might be solved by refining or upgrading the seed (seed upgrade). The I.D.S. method, developed by Milan Simak (1983) for conifer seeds, was evaluated for its effectiveness in refining thinleaf alder seed. I.D.S. involves imbibing the seeds, partially re-drying to leave a residue of moisture, and separating by a density method. The viable seeds should retain moisture while the non-viable should not, thus creating a density differential between viable and non-viable seeds. Thinleaf alder seed was subjected to simple density separation by the specific gravity method, using petroleum ether, with and without I.D.S. treatment. The I.D.S. method used was effective in eliminating empty seed of thinleaf alder, however, seed source influenced the effectiveness of the technique. Further research is necessary to determine the optimum duration of the "drying" time in the I.D.S. techniques.

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INTRODUCTION

New Mexico has two species of alder, Arizona alder (*Alnus oblongifolia* Torr.) which is found in the mountains of southwest New Mexico (Martin and Hutchins 1980, Vines 1960), and thinleaf alder (*Alnus tenuifolia* Nutt.), designated by Carter (1997) as *Alnus incana* ssp. *tenuifolia* Nutt., found in the northern and western mountains (Martin and Hutchins 1980, Vines 1960). Until recently, existence of these species has been of interest mainly from a botanical standpoint. However, with increasing land-use in the western United States, these trees may have a further purpose in the revegetation of degraded riparian areas, and as "oasis" plants for those interested in native landscapes (Phillips 1995).

Efficient propagation of nursery stock from seed requires extensive knowledge of the germination requirements and cultural methods needed for the particular species. Little is known about the propagation requirements for thinleaf alder. This deficit is due, in part, to a lack of demand for this species in the past. Extensive work has been done on the propagation of other species within *Alnus* , specifically those species of commercial value to the timber industry such as red alder (*A. rubra* Bong.). Information generated from propagation studies on these species has elucidated some universal seed characteristics and germination requirements for members of *Alnus*. Seeds are characteristically very small and light, and may have a winged integument to aid in wind dispersal. Average seed density for *A. tenuifolia* averages about 1488 seeds per gram (Vines 1960). Seed quality and germination capacity are often very low, as it is difficult to separate sound from empty seeds when size and weight are so low (Brinkman 1974, Schopmeyer 1974). Within species, germination requirements may differ with provenance (Fowler and Dwight 1964, Wilcox 1968) or even within a provenance (Bjorkbom et al. 1965, Schopmeyer 1974).

Methods to upgrade seed quality (separate viable from non-viable seeds) have been developed for different species. Conventional seed separation techniques are based on density, such as air column or liquid separation, or by size and shape, such as with screens. Separation of viable and non-viable seeds is extremely problematic with very light, winged seeds like those of alder. Air separation techniques may not be practical for winged, light-weight seed. Flotation techniques often employ lighter-than-water solvents, but some of these substances may have adverse effects on seed viability (Barnett 1971, McLemore 1965). Wide scale use of some solvents is not considered desirable because of health and safety concerns.

A method of seed refinement/upgrade originally developed by Milan Simak, called the I.D.S. method (Incubation, Drying, Separation) shows promise for separating live and dead seeds (cited in Bonner 1984, Downie and Wang 1992, Simak 1983, Sweeney et al. 1991). Seeds are imbibed for several hours, then incubated at cool temperatures (15°C) for several hours in 100% relative humidity. Seeds are then "dried" for several

hours at 35% relative humidity at cool temperatures (timing and relative humidity must be adjusted for the particular species). During the drying period, dead seeds will lose most of the water previously imbibed, while live seeds should retain most of their imbibed water. This differential moisture content would make separation by flotation and other density separation methods potentially feasible. Similar methods of conditioning have been shown to improve seed quality in lettuce, tomato, and onion (Hill et al. 1989). It has also been shown that drying of stratified seeds for storage or for separation from stratification medium need not result in loss of viability (Danielson and Tanaka 1978, Schopmeyer 1974).

The purpose of this study is to determine the effectiveness of the I.D.S. seed refinement technique and other separation procedures in increasing the percentage of live seeds in thinleaf alder seed lots. Secondly, this study examined the within-species variability of different seed lots in their response to I.D.S. and seed separation treatments.

METHODS AND MATERIALS

Seed Sources

Alder strobiles were collected in October and November of 1998 in Catron County, New Mexico, near the towns of Luna and Reserve, in the Cottonwood Canyon Campground and in the Head of the Ditch Campground; and in Taos County, New Mexico, in the Red River Canyon near the Molycorp molybdenum mine (Table 1). Strobiles were kept cool and allowed to dry for several weeks. Seeds were separated from the opening strobiles by rubbing on a coarse screen.

All seed sources were evaluated for percentage of filled seeds by means of dissection, performed under a dissecting microscope at 30X magnification (Berry and Torrey 1985). Alder species baseline percentage of filled seeds was estimated using 25 samples of 100 seeds pooled into one percentage response for each seed source. Baseline percentage fill (Table 1) is the estimate of the percentage of filled seed in the entire seed collection for each source.

Separation Media

Ethanol and water were not particularly effective in separation of thinleaf alder seeds, either using I.D.S. methods or when separating dry seed. It was necessary to choose a fluid with a lower specific gravity than ethanol (S.G.=0.79) in order to separate filled and empty seeds with very low densities. Falleri and Pacella (1997) found that low-density London plane tree (*Platanus* x *acerifolia* [Ait.] Willd.) seeds could not be separated using water as the separation medium due to the very small density differences between sound and empty seeds, and chose petroleum ether as a separation medium. Petroleum ether was chosen for the separation of thinleaf alder seeds because of its low specific gravity (S.G.=0.60), its relative stability, low reactivity, and rating as a slight health risk. Contact with skin may cause dryness and

Table 1. Seed Source Locations and Elevations

Species	Source	Lot No.	Baseline %Fill	Description	Elevation (meters)	Latitude Longitude
Thinleaf Alder	Luna	NA	23.4	Head of the Ditch CG	2134	N 33°49' W 108°59'
	Reserve	NA	26.8	Cottonwood Canyon	1829	N 33°37' W 108°55'
	RRC-1	98108	0.8	Red River Canyon	2469	N 36°41' W 105°29'
	RRC-2	98109	0.9	Red River Canyon	2469	N 36°41' W 105°29'

Table 2. Alder Preparation Protocols for Seed Refinement

Preparation Protocol	Imbibition Time (Hours)	Drying Time (Hours)
1- (Control)	0	0
2	24	0
3	24	1
4	24	18
5	24	24

irritation, but no chronic systematic effects have been reported with industrial use (Mallinckrodt Baker, Inc. 1997a).

Seed Refinement Treatments

Separation treatments examined included density separation of dry seed samples in petroleum ether (the control), and imbibed seed samples treated with the I.D.S. method at 0, 1, 18, and 24 hour drying times, followed by density separation in petroleum ether (Table 2). Seeds were imbibed for 24 hours by submersion in a 10-gallon glass aquarium filled with distilled water and equipped with an aeration pump and filter. Seeds were packaged in filter paper, then the packages were enclosed in wire cages (purchased tea balls were used for this purpose) weighted with marbles to keep them submerged. At the end of the imbibition period, seeds were removed from the cages, thoroughly blotted, and placed on clean filter paper. The drying incubation was performed in a closed chamber with a constant humidity obtained by the use of $CaCl_2$ $6H_2O$ salt in a saturated solution prepared by adding 5000g $CaCl_2$ $6H_2O$ to 3.0 liters of distilled water (Slavik 1974, Young 1967). Imbibed seeds were placed on filter paper and suspended on a screen above the calcium chloride solution. Humidity was monitored using an hygrometer, and held steady at 50% in the presence of the wet seeds and filter paper.

At the end of the appropriate drying incubation, the seeds were placed in petroleum ether and briefly and vigorously stirred to separate seeds adhering to one another. Floating seeds were removed from the surface of the petroleum ether by means of a small net and/or a spatula, placed on clean moistened filter paper and placed in a labeled plastic bag to await counting. The sinking seeds were strained through the net and packaged in a similar manner. Five repetitions were performed for each of the five treatments using 100 seeds per repetition. Percentage of filled seeds contained in each was determined by means of dissection tests performed on the floating and sinking fractions, using a scalpel and a dissecting microscope with 30X magnification.

In addition, the percentage recovery of filled seeds from the sinking fraction was calculated based on the total number of filled seeds present in that particular repetition:

$$\frac{\text{\# of filled seeds in the sinking fraction}}{\text{\# of filled seeds in the sinking fraction + \# of filled seeds in the floating fraction}} \times 100 = \text{percentage recovery}$$

Data Analysis

Percentage of filled seeds present in the sinking fractions (percentage fill), and proportion of filled seeds recovered from the total filled seeds available in the sample (percentage recovery) were response variables, and the preparation protocols and seed sources as independent variables. Data was analyzed by using categorical data modeling analysis as found in the SAS statistical program. The PROC CATMOD procedure can perform analysis and give "analysis of variance" in the general sense that it analyzes the response functions, fits linear models to functions of response frequencies, and partitions the variation among those functions into various sources (SAS Institute 1989).

All of the response variables considered had a binomial type of probability distribution (seed filled or not filled, seed germinated or not germinated). All treatments of both experiments were analyzed using the PROC CATMOD procedure to examine the general model, as well as planned comparisons using contrast statements where appropriate. The PROC MEANS procedure was used to calculate marginal percentages (main effect and interaction combinations), along with standard errors. Pairwise Z-tests were used to separate percentages in those effects which were determined to be significant by categorical modeling at the observed significance level, alpha = 0.05; this method of percentage separation is analogous to Fisher's LSD for separating means.

The categorical modeling procedure used two models, one for the percentage of filled seeds attained in the sinking and floating fractions, and one for the percentage of filled seeds recovered from those available in the baseline sample. The treatment structure for both of these seed refinement studies was a 5 X 2 X 4 factorial (preparation protocol by separation fraction by seed source).

The reader is referred to Jones (2000) for a more descriptive explaination of the categorical models used in this study.

RESULTS

Preparation protocol, seed source, and the separation fraction had significant (alpha=0.05) effect on the percentage fill (Table 3). The effect of separation fraction was influenced by both source and preparation protocol.

Protocols 1, 4, and 5, the control and 24 hour imbibition followed by either 18 or 24 hours drying, respectively, all had greater than 80% filled seed in the sinking fraction (Table 2, Figure 1). Twenty-four hour imbibition alone or in conjunction with 1 hour of drying both had lower percentages of filled seeds in the sinking fraction (less than 35%). Protocol 4, the 24-hour imbibition, followed by 18 hours of drying and density separation in petroleum ether, was chosen as the separation method for the germination requirements study.

Table 3. Analysis of Variance Table for Thinleaf Alder Percentage of Filled Seeds as Influenced by Preparation Protocol, Separation Fraction, and Seed Source--Factorial Analysis

Source	DF	Chi-Square	Observed Significance Level
Intercept	1	116.63	0.0000
Seed Source	3	173.67	0.0000
Preparation Protocol	4	44.90	0.0000
Separation Fraction	1	88.29	0.0000
Source*Prep	12	5.41	0.9427
Source*Fraction	3	9.71	0.0212
Prep*Fraction	4	9.86	0.0429
Source*Prep*Fraction	11	7.14	0.7878

Table 4. Thinleaf Alder Percentage of Filled Seeds in the Fractions as Influenced by Source and Compared to Baseline % Fill Uninfluenced by Preparation Protocol

Seed Source	Baseline % Fill	% Fill - * Sinking Fraction	S.E.	% Fill - * Floating Fraction	S.E.	*N*
Luna	23.4	86.34c	1.80	12.65d	0.72	4000
Reserve	26.8	46.44b	1.39	6.31c	0.70	4000
RRC-1	0.8	4.44a	1.31	0.44a	0.14	4000
RRC-2	0.9	9.09a	3.28	0.62a	0.15	4000

*Percentages followed by the same letter are not significantly different at α=0.05.

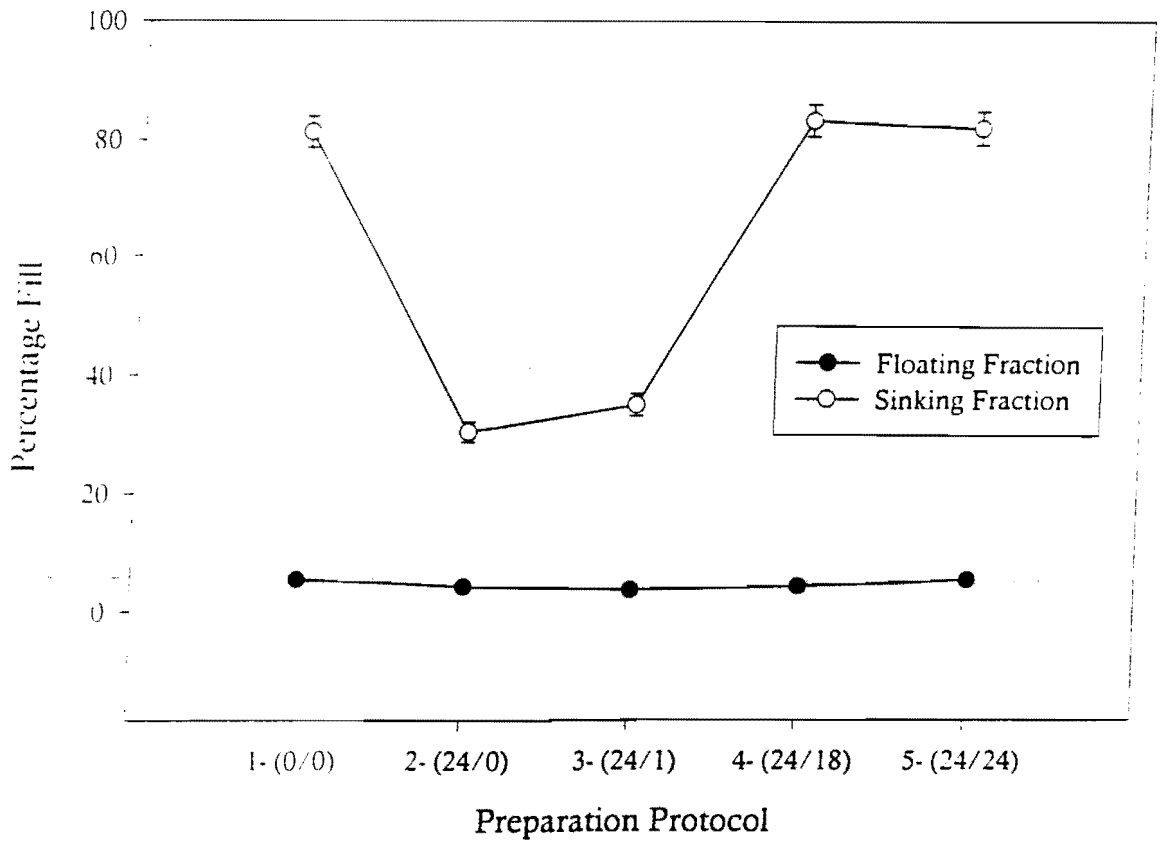

Figure 1. Alder Percentage Fill as Influenced by Preparation Protocol and Separation Fraction. Error bars represent +/- one Standard Error. Bars which are not visible are smaller than the symbol used to represent the percentage. (Protocols are described in Table 2).

The proportion of filled seed in the sinking and floating fractions was also influenced by seed source. Percentage of filled seeds in the sinking fraction ranged from 4.44% for the Red River Canyon #1 source to over 86% for the Luna source (Table 4). Percentage of filled seeds in the floating fraction ranged from less than 1% to just over 12%, while the baseline percentage of filled seeds in the seed sources ranged from less than 1% to over 26%. The separation process improved percentage fill in the sinking fraction compared to the percentage fill in the floating fraction by about seven-fold for the Luna and Reserve seed sources, ten-fold for the Red River Canyon #1 source, and almost fifteen-fold for the Red River Canyon #2 source. Separation improved the percentage of filled seeds in the sinking fraction compared to the unseparated seed source by almost four-fold for the Luna source, almost two-fold for the Reserve source, almost six-fold for the Red River Canyon #1 source, and ten-fold for the Red River Canyon #2 source.

Floating separation fractions had a much lower percentage of filled seeds (4.64%) than sinking fractions (47.11%) (Table 5). Percentage of filled seeds was consistently low in the floating fraction but varied with the preparation protocol in the sinking fraction (Figure 1).

Seed source and preparation protocol both influenced the percentage of filled seeds recovered (Table 6). In contrast to the percentage of filled seeds in the sinking fraction (Figure 1), the percentage of seeds recovered was improved by 24 hours imbibition alone or with one hour drying at 50% humidity (Table 7). These two treatments had in excess of 80% recovery whereas the other three separation treatments all averaged less than 67% recovery.

Percentage recovery as influenced by source varied from approximately 32% for the Red River Canyon #2 collection to over 88% for the Reserve seed source (Table 8). The Red River Canyon #1 and Luna sources both had percentage recoveries slightly greater than 50%.

DISCUSSION

Traditionally seed refinement has been thought of as enhancing the number of potentially viable seeds (filled seeds) in a seed lot. Previously published studies have used total germination as the measure of seed refinement efficacy. In this study the number of filled seeds in the sinking fraction was used. The I.D.S. treatments imposed did not improve the number of filled seeds in the sinking fraction in comparison with ordinary gravity separation. In two of the alder I.D.S. treatment levels, 24-hour soak with either no drying time or one hour of drying time actually reduced the percentage of filled seeds in the sinking fraction. The two remaining alder I.D.S. treatments had considerably longer drying times and resulted in percentages of filled seeds in the sinking fraction similar to those of the non-imbibed control treated by gravity separation. The influence of drying time on the efficacy of the I.D.S. treatment has

Table 5. Thinleaf Alder Percentage of Filled Seeds as Influenced by Separation Fraction

Separation Fraction	% Fill *	S.E.	*n*
Floating Fraction	4.63a	0.23	8030
Sinking Fraction	47.11b	1.12	1970

*Percentages followed by the same letter are not significantly different at α=0.05.

Table 6. Analysis of Variance Table for Thinleaf Alder Percentage of Filled Seeds Recovered in the Sinking and Floating Fractions as Influenced by Preparation Protocol and Seed Source--Factorial Analysis

Source	DF	Chi-Square	Observed Significance Level
Intercept	1	6.94	0.0084
Seed Source	3	110.55	0.0000
Preparation Protocol	4	23.71	0.0001
Source*Prep	12	17.15	0.1439

Table 7. Thinleaf Alder Percentage of Filled Seeds Recovered in the Sinking Fraction as Influenced by Preparation Protocol

Protocol (Soak/Dry)	% Recovery *	S.E.	*n*
1 - 0/0	64.47a	2.90	273
2 - 24/0	80.94b	2.36	278
3 - 24/1	82.25b	2.30	276
4 - 24/18	66.67a	3.12	228
5 - 24/24	60.41a	3.12	245

*Percentages followed by the same letter are not significantly different at α=0.05.

Table 8. Thinleaf Alder Percentage of Filled Seeds Recovered In the Sinking Fraction as Influenced by Seed Source

Seed Source	%Recovery *	S.E.	*n*
Luna	53.92b	2.06	586
Reserve	88.52c	1.23	671
RRC-1	52.38ab	10.90	21
RRC-2	31.82a	9.93	22

*Percentages followed by the same letter are not significantly different at α=0.05.

been seen in other species (Falleri and Pacella 1997, Sweeney et al. 1991). In a study of London plane tree, researchers found that as drying time increased from 7.5 hours to 24 hours observed germination percentage was greater than control (Falleri and Pacella 1997). At drying times less than 7.5 hours, observed germination was comparable to unseparated controls. In the same study, only seed receiving 24 hours of drying as part of an I.D.S. treatment had greater germination than non-treated seed separated in petroleum ether.

The response of the alder seed to I.D.S indicates there may be potential for I.D.S. as a seed refinement tool, using longer imbibition and drying times. The difference in times from the 1-hour to the 18-hour drying is considerable and corresponds to a significant difference in the percentage of filled seeds in the sinking fraction. The shorter drying times may have been of insufficient duration to allow the unfilled seed to lose sufficient moisture and hence these seeds ended up in the sinking fraction. In contrast, the 18- and 24-hour drying times may have allowed the imbibed unfilled seeds to lose the majority of the water imbibed, and resulted in percentages of filled seeds in the sinking fraction similar to those seen in the non-imbibed controls.

While all thinleaf alder sources had improved percentages of filled seeds in the sinking fractions, there appear to be differences between sources in response to seed refinement. This difference was most pronounced when comparing the Reserve and Luna seed lots. The Luna seed lot had over a three-fold improvement in the percentage of filled seeds in the sinking fraction compared to the percentage of filled seeds in the initial seed lot (23.4%). In contrast, seeds from the Reserve seed source had a similar initial percentage of filled seeds (26.8%) but the percentage of filled seeds in the sinking fraction was slightly less than twice this amount (46.4%). Differences of seed source in response to similar seed refinement techniques (I.D.S.) has been observed in other studies (Donald 1985, Downie and Wang 1992). Downie and Wang (1992) found that the I.D.S. treatment improved germination percentage regardless of the initial seed lot germination capacity in three conifer species, lodgepole pine (*Pinus contorta* var. *latifolia* Engelm.), Jack pine (*P. banksiana* Lamb.), and white spruce (*Picea glauca* [Moench] Voss). The authors of that study did not compare responses between seed sources. While not tested in this study, there may have been differences in the rate of moisture loss between the Luna and Reserve sources during the drying portion of the I.D.S. regimes imposed in this study. More detailed studies examining source differences in the rate of moisture loss would be beneficial.

The above discussion focuses primarily on reducing the number of empty or non-viable seeds in a seed lot. During seed refinement some viable seed is also lost in the floating fraction (Downie and Wang 1992, Falleri and Pacella 1997, Sweeney et al. 1991). In cases where there is more than adequate seed supply, the loss of viable seed in the floating fraction is not a problem. In those cases where the amount of available viable seed is limited and losses of viable seeds needs to be minimized, other criteria

can be used to determine the most effective seed refinement technique. Such was the case in this study.

The percentage of filled seeds recovered in the sinking fraction provides a measure of how efficient the refinement technique is at reducing the number of filled (potentially viable) seeds lost in the floating fraction. In the current study involving alder, those protocols with low percentages of filled seeds in the sinking fraction had a high percentage of filled seeds recovered (Figure 2). In the case of alder, the high recovery of filled seeds was inversely related to the I.D.S.
treatment's ability to remove non-viable seed. A similar trend was observed in another study in an attempt to upgrade germinated cabbage seeds using density gradients. As percentage recovery increased, the percentage of germinated seeds decreased because of the increased recovery of non-germinated seeds (Taylor and Kenny 1985). The technique employed to determine which seed refinement protocol to use in the germination studies was to multiply the percentage of filled seeds in the sinking fraction by the percentage of filled seeds recovered. This value addresses both the protocol's ability to remove non-viable seeds as well as its ability to reduce the loss of potentially viable seeds.

Depending on a grower's constraints, either greenhouse space or seed supply, the evaluation of a seed refinement technique could be based on one of three criteria discussed above: percentage of filled seeds in the sinking fraction, percentage of filled seeds recovered, or the product generated by multiplying these two values as was done in this case. In cases where seed supply is a greater constraint, selection of seed refinement technique may be based solely on the percentage of filled seeds recovered. This seed refinement technique may not be as efficient in removing unfilled seeds, but loss of filled seeds would be minimized. In the case where growing space is the greater constraint, the percentage of filled seeds in the sinking fraction would be the criteria used for seed refinement technique selection. If both greenhouse space and seed supply are limited, then the product of the two may be used to determine the appropriate protocol. The use of this information in conjunction with spreadsheet-based seed sowing programs allows nursery managers to select the best seed refinement technique for their nursery (Harrington and Glass 1997, Wenny 1993).

The particular separation medium found to be most effective will vary with species. Large and dense seeds may often be effectively separated using water as the medium (Simak 1983). This is known as the specific gravity method of separation when used on untreated seeds. In very small seeds where the density gradient between empty, dead, and filled live seeds is not great, water may not be effective, and it is more advantageous to adjust the specific gravity of the separation medium, rather than trying to make fine adjustments in the density gradient of the seeds to be separated (Downie and Wang 1992).

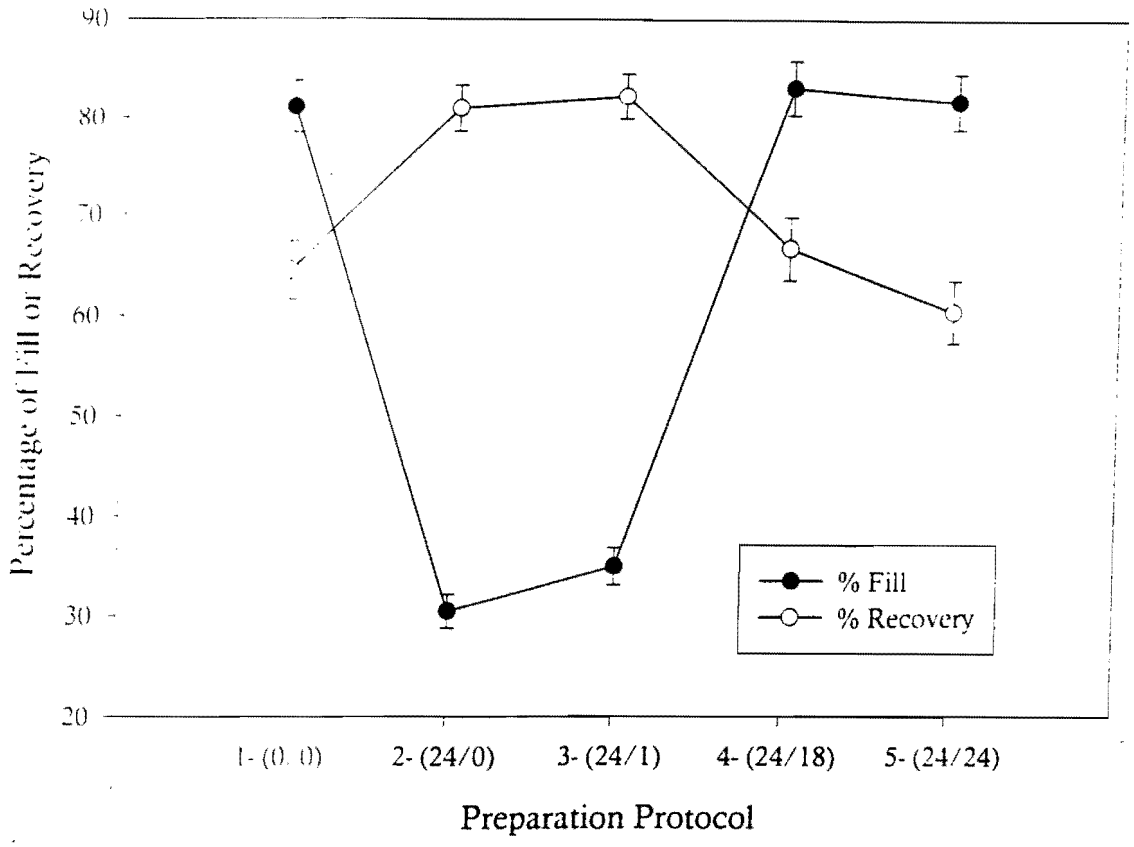

Figure 2. Alder Percentage Fill and Percentage Recovery of the Sinking Fraction as Influenced by Preparation Protocol. Error bars represent +/- one Standard Error. (Protocols described in Table 2).

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