

Polyamines and Inorganic Ions Extracted from Woody Tissues by Freeze-thawing¹

Rakesh Minocha and Walter C. Shortle²

ABSTRACT

A simple and fast method for extraction of major inorganic ions (Ca, Mg, Mn, K, and P) and cellular polyamines from small quantities of wood and woody plant tissues is described. The method involves repeated freezing and thawing of samples instead of homogenization or wet ash digestion. The efficiency of extraction of both polyamines and inorganic ions by these methods was compared for 10 different tissues. Drillbit shavings generated from wood disks or increment cores were also compared with ground wood as the starting material for ion analysis by this newly developed method of freeze-thawing. Direct use of drillbit shavings circumvents the need for making wood chips by hand and grinding in a Wiley mill. Moreover, freeze-thawing not only eliminates the need for various tissue homogenizers but is also simple enough that a large number of samples can be processed simultaneously. This method seems to be particularly useful with extremely small samples of 25 mg or less, e.g., shavings from individual growth rings of mature trees and differentiating tissues grown in vitro.

INTRODUCTION

Mobilization patterns of one or more ions within wood can be related to either wood decay processes (Safford and others 1974) or environmental stress conditions (Blanchard and others 1978; Bondiotti and others 1989, 1990; Shortle and Smith 1988) in living trees. Also, the yearly variations in the inorganic ion content of a tree ring or increment core may be indicative of the composition of the nutrients taken up by the tree during that growth period (Bondiotti and others 1989, Pillay 1976). Thus, changes in the ion composition of wood and woody plant tissues grown in culture may be used to evaluate the current growth potential and/or predict vulnerability of trees to environmental stress, injury, and infection.

Polyamines spermidine, spermine, and their precursor, putrescine, have been found to play a significant role in the growth and development of plant cells (Slocum and Flores 1991, Smith 1985). The cellular polyamine content is highly regulated. A variety of stimuli including Ca and Mg deprivation (Smith 1973), pathogenesis (Greenland and Lewis 1984), ozone and acid stress (Dohmen and others 1990), aluminum stress (Minocha and others 1992), etc., all lead to an accumulation of one or more of the polyamines. Divalent cations such as Ca and Mg have been shown to substitute for polyamines in some of their metabolic activities, especially under stress conditions (Minocha and others 1992, Smith 1985). This information has prompted numerous studies on the quantitative analysis of cellular polyamines in various plant tissues (Minocha and others 1990, Smith 1991).

Most of the published work on extraction of polyamines from various tissues involved homogenization of tissue in perchloric acid (PCA) or trichloroacetic acid (TCA) using one of the following: (a) A chilled mortar and pestle with liquid nitrogen; (b) a polytron or tissumizer; or, (c) conical ground glass homogenizers (Birecka and others 1988; Faure and others 1991; Kushad and Yelenosky 1987; Maki and others 1991; Meijer and Simmonds 1988; Minocha and others 1991, 1992, 1993; Nielsen 1990; Rastogi and Davies 1989; Torrigiani and others 1987). These methods of grinding, though not very complicated, are time consuming and often noisy due to the use of polytron for long periods. Similarly, commonly used extraction procedures of dry and wet ash digestion for the determination of total inorganic ions require a rather large sample size (100 to 1,000 mg) and are time consuming, laborious, costly, and in many cases, hazardous (Anderson and Henderson 1986, Isaac and Johnson 1976, Kingston and Jassie 1986, Kuennen and others 1982, Wikoff and Moraghan 1986, Wolf 1982). In most cases, it is difficult to process a large number of samples by any of these methods. A quick and safe procedure for extraction of cellular-free polyamines and exchangeable or total inorganic ions would be useful to many laboratories.

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² USDA Forest Service, Northeastern Forest Experiment Station, Concord and Mast Roads, Durham, NH 03824.

Extraction by repeated freeze-thawing as a reliable method for quantification of cellular polyamines as well as inorganic ions from woody plant tissues is presented. Homogenization of wood was also tested along with freeze-thawing to evaluate whether this procedure could also replace wet ash digestion for extraction of inorganic ions.

METHODS

Tissue Preparation

Wood

For ground wood samples, sapwood of air-dried disks taken from several mature red spruce (*Picea rubens* Sarg.) trees was chipped and ground in a Wiley mill to pass through a 420- μ m sieve. This pooled ground wood was mixed thoroughly and used as an inhouse reference material since no standard reference material for nutrient content of wood was available from the National Institute of Science and Technology (NIST) for use in method development and quality control.

As an alternative to grinding, a relatively fast and effective method of drilling was developed to prepare wood samples in small quantities either from red spruce or red oak (*Quercus rubra* L.) wood disks or from tree ring cores of red spruce. Briefly, the wood surface of an increment core was cleaned by drilling a 1.0- to 3.0-mm deep hole with a 3.2-mm cobalt twist bit. A 7.9-mm titanium twist bit was used for cleaning of 5.0-cm thick air-dried disks. The shavings generated were dusted off the surface and discarded. At this point, either a 3.2-mm or 6.4-mm cobalt drill bit was used to collect either fine or coarse shavings (fig. 1). All the shavings of a certain size were pooled to provide homogenous material for method comparison (for details, see Minocha and Shortle 1993). All wood samples were oven-dried at 80 °C for 16 to 24 h and cooled to room temperature over silica gel in desiccators before weighing. Five replicates were used for each treatment unless stated otherwise. Polyamine analyses were not performed for wood samples.

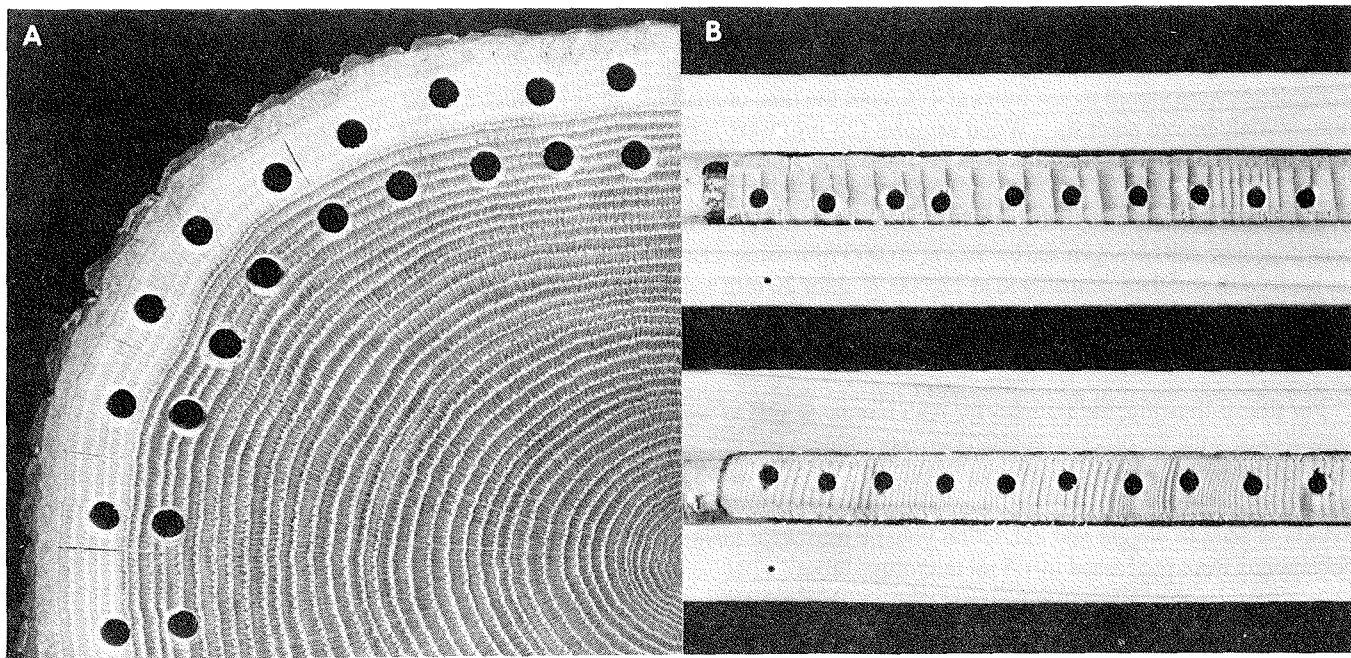


Figure 1.-(A) An oak wood disk showing the arrangement for taking sapwood and heartwood samples using 6.4-mm-diameter drill bit. (B) 12-mm-diameter increment cores from red spruce glued to grooves in wood blocks. A 3.2-mm-diameter drill bit was used to drill holes within single annual rings of a fast-growing tree (Top) or at a set distance regardless of number of rings per hole (Bottom).

Needles, Callus Tissue, and Cell Suspensions

Tissues were prepared in one of the following ways: (a) Needles from one or more red spruce seedlings were pooled, washed with distilled water, blot dried, and finely chopped; (b) callus tissue grown on agar solidified medium was pooled from several plates, blotted on filter paper to remove excess moisture and finely chopped before weighing; and (c) suspension cultures from two or more flasks were mixed, collected on Miracloth (Calbiochem-Behring Corp., La Jolla, CA), and thoroughly washed with 3 volumes of deionized distilled water. Aliquots were taken from these pooled tissues for different methods as well as for replicates within the same method.

The tissues tested included: callus of Norway spruce, *Picea abies* L. (Karst.), and aspen, *Populus tremuloides*; cell suspensions of red spruce, *Picea rubens* (Sarg.); hybrid poplar, *Populus nigra* X *P. maximowiczii*, and periwinkle, *Catharanthus roseus*; needles (1-year-old, 2-year-old, or mixed) and roots of 2-year-old red spruce seedlings. Whole dry needles from mature red spruce trees were also tested. Tissue samples were replicated four to five times unless otherwise indicated.

Wet Digestion

For wet ash digestion (W), the procedure of Isaac and Johnson (1976) as modified by Michaelson and Ping (1990) was followed. Briefly, 200 ± 0.5 mg of well-mixed sample was transferred to a 75-ml block digest tube followed by the addition of 7 ml of digestion mixture (97 g of selenous acid dissolved in 100-ml ultrapure [Millipore Corporation, Bedford, MA] water and added slowly to 4 kg bottle of concentrated sulfuric acid). Two Teflon boiling stones and 0.5 ml of 50 percent hydrogen peroxide were added to the digestion mixture in each tube. The tube was vortexed and placed in a preheated (400 °C) block for 30 sec and then removed. The step of adding 0.5 ml hydrogen peroxide and heating for 30 sec was repeated until the solution became clear (about 4 ml). Before hydrogen peroxide was added, the tubes were removed from the heating block to allow them to cool for 1 min to avoid loss of volume due to effervescence. Once the solutions were clear, the digestion was allowed to continue for another 55 min (total digestion time 60 min). After the tubes were cooled to room temperature, ultrapure water was added to bring the volume to 75 ml, and the solution was transferred to acid-cleaned storage bottles until ready for analysis. Two blanks were digested per batch of samples.

Extraction by Homogenization

For the extraction of inorganic ions by homogenization, 5 ml of 0.01 N HCl were added to a 50-mg tissue sample in a 15-ml acid-washed glass test tube. The samples were homogenized for 90 s at 20,000 rpm using a Brinkmann Polytron homogenizer. The extracts were stored in test tubes at 4 °C until the time of analysis. Samples were filtered using a 45- μ m nylon syringe filter immediately before analysis. Wood samples (25 mg) were homogenized for 90 s at 24,000 rpm with Teckmar tissumizer or Brinkmann polytron in 1 ml of 0.01 N HCl and final volume brought to 5 ml (Minocha and Shortle 1993).

For polyamine analysis, 200-mg tissue samples were transferred to 5 percent PCA (tissue:PCA ratio 1:4) in a 15-ml Corex centrifuge tube and homogenized for 90 s at 20,000 rpm using a Brinkmann Polytron homogenizer. The samples were incubated on ice for 1 h and then centrifuged at $18,000 \times g$ for 20 min and the supernatant kept frozen at -20 °C until dansylation.

Extraction By Freeze-thawing

For freeze-thawing, the samples for both inorganic ions as well as for polyamines were frozen at -20 °C and thawed at room temperature, with the process repeated two more times. The duration of the freezing step varied from 4 h to a few days. Samples were allowed to thaw completely (not to exceed 1.5 h for tissues grown in culture and 5 h for wood samples) before refreezing. After freeze-thawing, the samples were either filtered (for inorganic ions) or centrifuged at $13,500 \times g$ (for polyamines). Other details of sample weight and extraction volume were kept identical to the homogenization method except that the samples for polyamines were processed in 1.5 ml microfuge tubes.

Ion Analysis

The concentrations of major inorganic ions were determined by Beckman Spectra span V ARL DCP-AES (Direct Current Plasma Atomic Emission Spectrometer, Beckman Instruments Inc., Fullerton, CA) using the Environmental Protection Agency's (EPA) method number 66-AES0029 (1986).

Polyamine Analysis

Prior to dansylation, heptanediamine was added to the extracts as an internal standard for polyamine analysis. Fifty microliters of the extract were dansylated, separated by reversed phase HPLC (Perkin-Elmer Corp., Norwalk, CT) using a gradient of acetonitrile and heptanesulfonate and quantified by a fluorescence detector (Minocha and others 1990).

Statistical Analysis

Wood

Cochran's test for homogeneity of variance was performed on data sets involving comparison of more than two treatments (Cochran 1941). In cases where the null hypothesis of homogeneity was accepted, analysis of variance was carried out using SAS version 6.7 (SAS Institute Inc., Cary, NC). If the treatment differences were significant, Duncan's multiple range test was performed to separate the multiple treatment means (Duncan 1955). In cases where the null hypothesis of homogeneity was rejected, a t-test for unequal variances was performed comparing each treatment separately with the standard treatment. For data sets involving only two treatments, Satterthwaite's t-test for equal or unequal variances was performed using the same version of SAS.

Needles, Callus Tissue, and Cell Suspensions

Systat 5.02 (Systat Inc., Evanston, IL) for windows was used to perform either one way analysis of variance or a t-test for independent samples in order to evaluate if the treatment means were significantly different (two-tailed $\alpha = .05$). An f-test for homogeneity of variance was performed on data to determine if a t-test for equal or unequal variances should be used (Snedecor and Cochran 1956). In cases where the null hypothesis of homogeneity was accepted, pooled variances were used for t-tests. In cases where the null hypothesis of homogeneity was rejected, a t-test for separate variances was performed.

RESULTS

Inorganic Ions

Wood

Initially, both homogenization and freeze-thawing methods were tested on the inhouse reference material (ground red spruce sapwood). The results presented in figure 2 show that there was no significant difference between amounts of Ca, Mg, and Mn extracted by any of the three methods. However, the amount of K extracted by freeze-thawing was significantly higher as compared to wet digestion and homogenization. As both fine and coarse drill shavings of red spruce and red oak, collected by drilling, were equally effective as the starting material for extraction of these ions, data are presented only for coarse shavings. The yield of Mn from red spruce sapwood and heartwood shavings was significantly higher with freeze-thawing as compared to wet digestion and homogenization (fig. 2).

In oak sapwood, Mg was fully extractable by any of the three methods. However, Mn, which was present in minute quantities, was fully extractable only by freeze-thawing and wet digestion. Calcium extraction was consistently reproducible but not complete by either homogenization or freeze-thawing (fig. 2). In oak heartwood, both Mn and Mg were present in barely detectable levels and only about 85 to 90 percent Ca was extracted by freeze-thawing or homogenization as compared to wet digestion. However, heating the tubes at 95°C for 1 h extracted all of the Ca (Minocha and others 1993). Similar to the situation with red spruce wood, K was extractable in significantly higher quantities by freeze-thawing from both sapwood and heartwood of red oak (fig. 2).

Needles, Callus Tissue, and Cell Suspensions

Whereas P could not be extracted reliably from wood, its extraction by freeze-thawing was equal to or better than that by homogenization with the exception of aspen tissue (fig. 3). Likewise, the freeze-thawing method extracted equal or significantly higher amounts of Ca, Mg, and Mn as compared to homogenization from Norway spruce callus, hybrid poplar cell suspensions, red spruce roots, and 1-year-old needles (fig. 4). Aspen callus yielded slightly lower but not significantly different amounts of all the five ions with freeze-thawing in comparison with homogenization. In the case of periwinkle and red spruce cell suspensions also, the yield of both Ca and Mn was reproducible but somewhat lower by freeze-thawing (fig. 4).

Polyamines

Freeze-thawing extracted equal or significantly higher amounts of free putrescine and spermidine from all the tissues tested (fig. 5). Spermine was present in relatively small quantities in most tissues except for red spruce cell suspensions. It was also extracted equally well with freeze-thawing as compared to homogenization (fig. 5). The HPLC profiles by either method were similar; i.e., no peaks were missing from the extracts in either case.

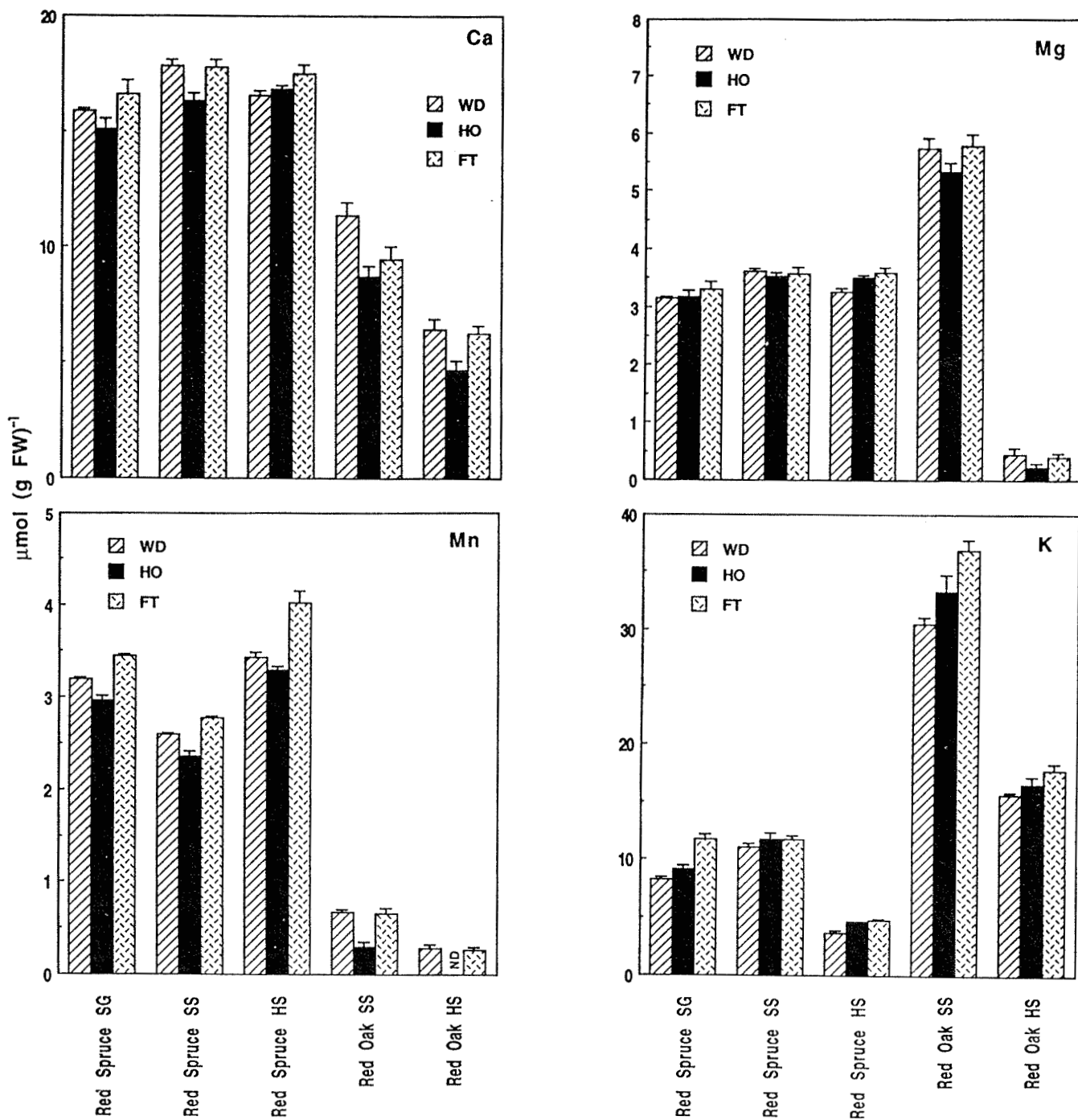


Figure 2.-Comparison of wet digestion (WD), homogenization (HO), and freeze-thawing (FT) for extraction of major inorganic cations from woody tissues. SG=sapwood ground; SS=sapwood coarse shavings; and HS=heartwood coarse shavings. Data are mean $\pm$ SE of five replicates.

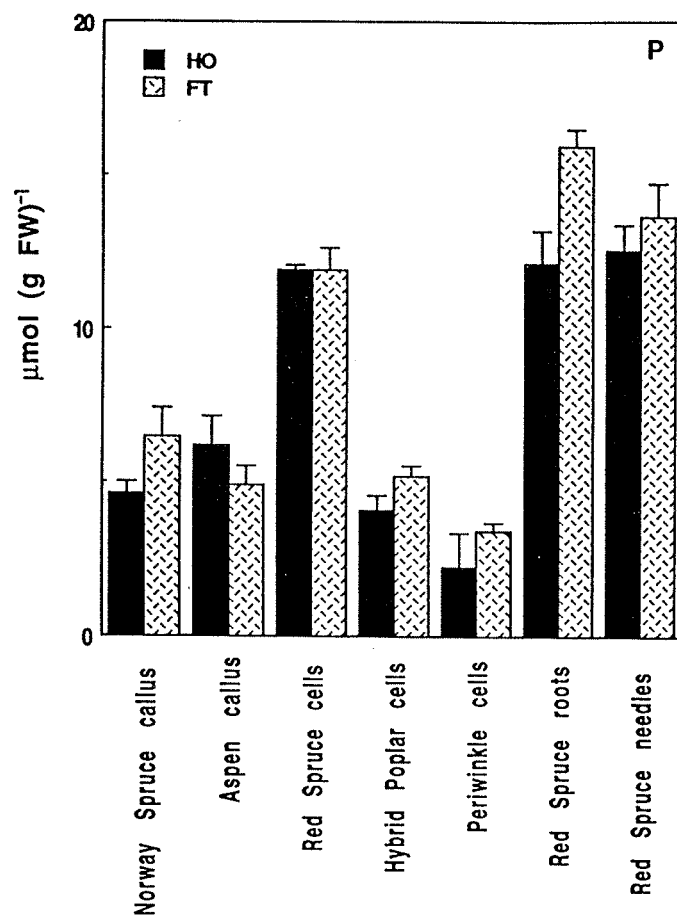


Figure 3.- Comparison of homogenization (HO) and freeze-thawing (FT) for extraction of P from various nonwoody tissues. Data are mean $\pm$ SE of four replicates for homogenization and five replicates for freeze-thawing.

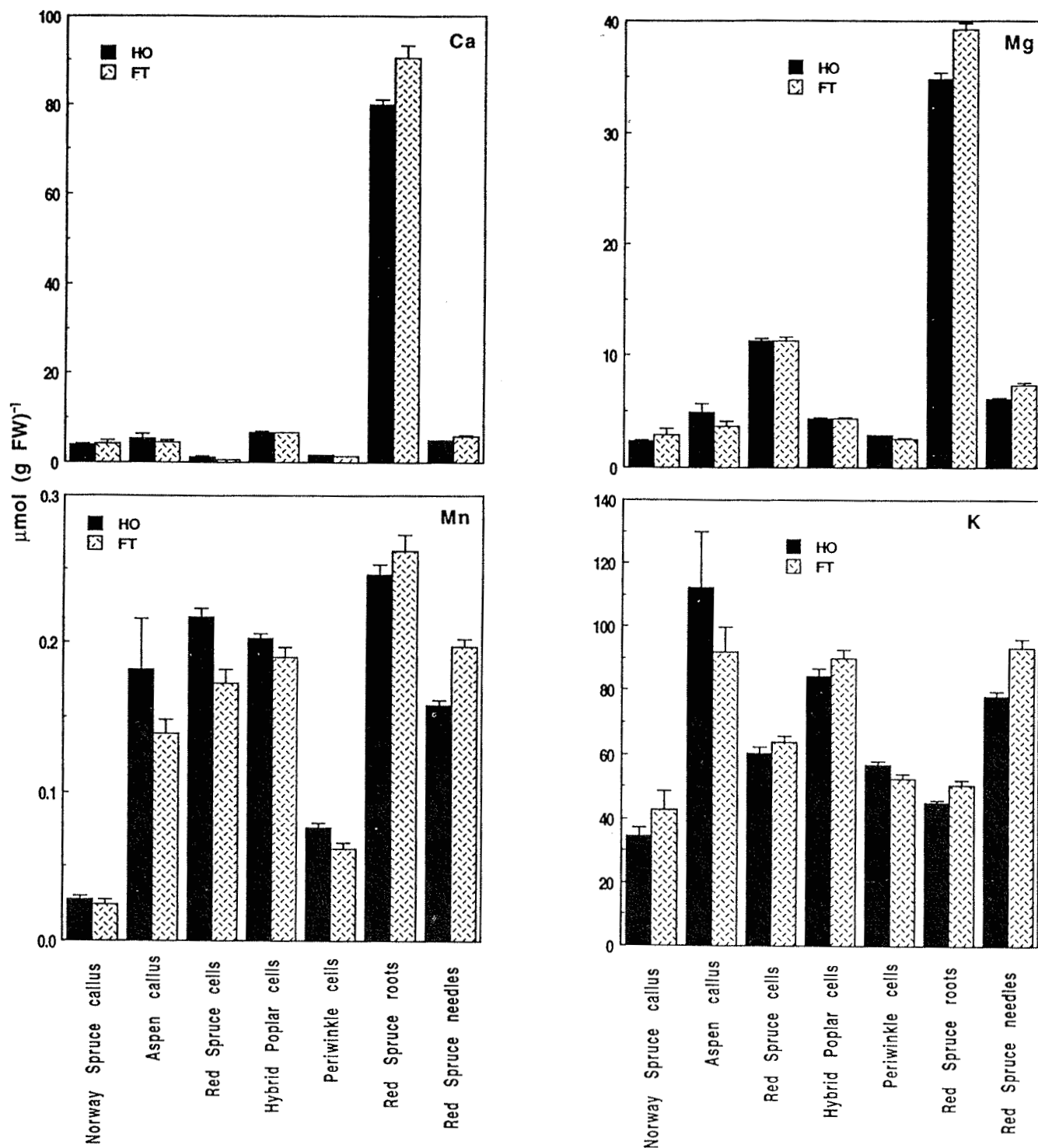


Figure 4.-Comparison of homogenization (HO) and freeze-thawing (FT) for extraction of major inorganic cations from various nonwoody tissues. Data are mean $\pm$ SE of four replicates for homogenization and five replicates for freeze-thawing.

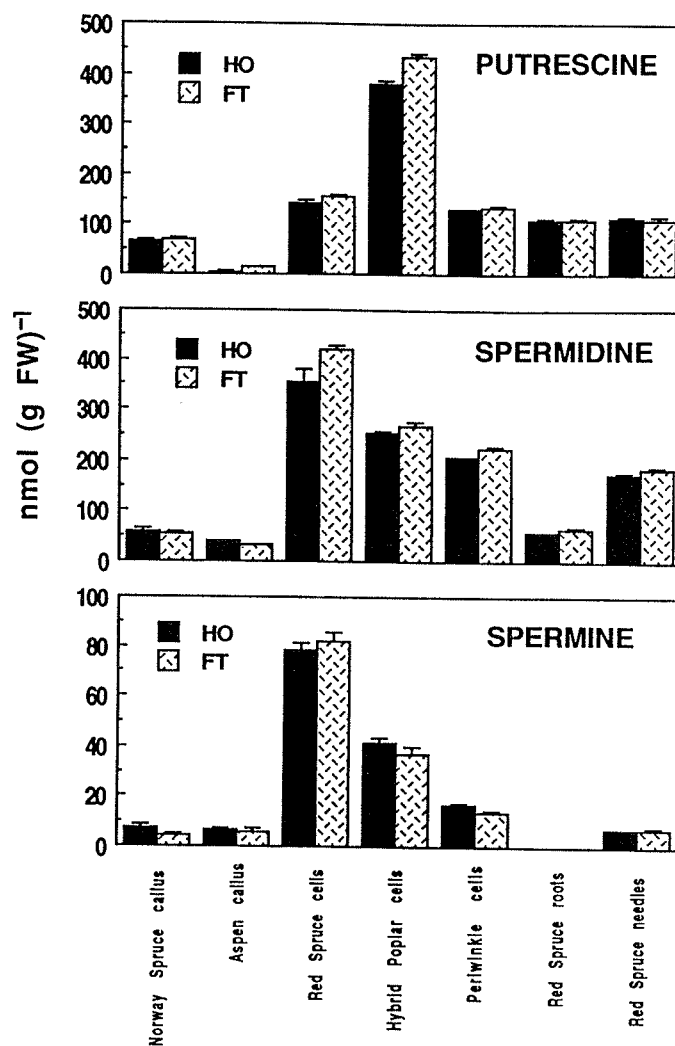


Figure 5.-Comparison of homogenization (HO) and freeze-thawing (FT) for extraction of putrescine, spermidine, and spermine from various nonwoody tissues. Data are mean $\pm$ SE of five replicates.

DISCUSSION

Wood

The use of drill-bit shavings directly for digestion of samples or for other extraction procedures has several advantages over using ground wood. It eliminates the need for chipping and milling the wood. As compared to Wiley mills, the drill-bits are easier to clean in between samplings. The samples for iron can also be analyzed by using non-ferrous drill-bits. Most importantly, they provide a fast way to study the historical record within a tree by drilling within individual tree rings of a core for fast-growing trees or within a fixed distance or decade for slow-growing trees (fig. 1).

Some of the major disadvantages of commonly used procedures of wet digestion of wood samples are: requirement for special equipment, inability to handle large numbers of samples, requirements for large dilutions that limit the analysis of micronutrient, and potential hazards of chemical explosion. In contrast, the freeze-thawing method is simple, is applicable to small amounts of tissue, and allows the processing of a large number of samples at the same time. Above all, it does not require a fume hood, complex temperature treatments, or special equipment.

Higher levels of K were consistently recovered by freeze-thawing and homogenization as compared to wet digestion. One possible explanation for this could be an earlier observation by Jackson (1962) that higher temperature during extraction by wet digestion causes volatilization of K.

Needles, Callus Tissue, and Cell Suspensions

Homogenization of tissue samples, though routinely used in most laboratories, is a relatively slow extraction procedure due to the fact that each sample must be handled separately. Freeze-thawing, on the other hand, is amenable to batch processing of a large number of samples. In our laboratory, 50 to 100 samples at a given time can be routinely processed. Moreover, no special equipment is needed. The yield of major inorganic ions by either homogenization or freeze-thawing, though repeatable, may or may not represent total ions present in the tissue. It is expected that both procedures would extract only the exchangeable or soluble ions from the tissue. Total ions from fresh or dry herbaceous tissues are usually extracted by wet or dry ash digestion. The extraction of ions from fresh needles of 1-year-old seedlings of red spruce by freeze-thawing, homogenization, and wet digestion were compared and it was found that all three methods provided complete extraction of the four ions under study. However, it was not true for dried whole needles of mature red spruce trees (Minocha and Shortle 1993). It is known that highly immobile, water-insoluble forms of major inorganic ions, such as calcium carbonate and calcium oxalate, occur in older or infected tree tissues. To mobilize ions in these forms requires increased acidity or heat, as in the case of oak heartwood (Minocha and Shortle 1993). Thus, if the goal is to study changes in exchangeable or non-covalently bound portions of ions in relation to a particular stress or a change in metabolic state of the tissue, freeze-thawing with 0.01 N HCl may be quite appropriate to use.

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