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SEASONAL VARIATION OF ISOZYMES IN JUNIPERUS SCOPULORUM: SYSTEMATIC SIGNIFICANCE[1]

WALTER A. KELLEY* AND ROBERT P. ADAMS[2]
Department of Botany and Plant Pathology, Colorado State University, Fort Collins 80523

A B S T R A C T

The relationship of seasonal variation in isozymes to systematic studies at the infraspecific level is evaluated. Isozyme variation in peroxidases, esterases, and α-terpineol dehydrogenases was evaluated monthly for one year in *J. scopulorum* Sarg. Zymograms of α-terpineol dehydrogenases showed one nonvariable band. Isoperoxidases varied quantitatively but not qualitatively, this variation being correlated with seasonal growth and dormancy. Isoesterases showed qualitative variation, with two types of esterases being produced. One type showed seasonal variation and a second was nonvariable. The presence of two types of isoesterases may reflect differing physiological roles for each. The esterase results showed that care should be taken during investigations of isozymes at the population level to assure phenological similarity.

THE STUDY of isozymes is common in systematic botany, especially at the infraspecific level. The majority of these investigations have dealt with the nature of genetic polymorphisms in annual herbaceous angiosperms with relatively few studies in the gymnosperms (Kelley, 1976). This bias is in part the result of the great difficulty in obtaining active enzyme extracts from plants with high phenol/protein ratios (Kelley and Adams, 1977a).

Isozyme studies in conifers have dealt with several areas of investigation. The genetic control and inheritance of isozymes have been studied to a considerable degree in *Picea abies* (Bartels, 1971; Lundkvist, 1974, 1975) and *Pinus attenuata* (Conkle, 1971a, 1971b). Analysis of marginal and central populations of *P. abies* showed a large amount of genetic variability (Tigerstedt, 1973). Genetic differences and populational variation have also been examined in *Cryptomeria japonica* (peroxidase, Sakai and Park, 1971), *Pinus attenuata* (alcohol dehydrogenase, and leucine amino peptidase, Conkle, 1971a), *Thujopsis dolabrata* (peroxidase, Sakai, Miyazaki, and Matsuura, 1971), *Pinus pungens* (alcohol dehydrogenase, and leucine aminopeptidase, Feret, 1974), *Pseudotsuga menziesii* (peroxidase, Muhs, 1974) and *Juniperus ashei* (alcohol dehydrogenase, esterase, and peroxidase, Kelley and Adams, 1977b). The aforementioned studies largely utilized collections from plants in natural environments.

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[2] Present address: Science Research Center, Box 1095, Gruver, Texas 79040.

The goal of many isozyme investigations has been to evaluate the patterns of variation which occur under natural conditions and to attempt to correlate them with some selective force or forces. Systematic studies which try to evaluate the nature of this variation throughout the range of perennial species may be subject to errors due to seasonal variations (Adams, 1970; Powell and Adams, 1973). This may be most pronounced when the range of the species studied varies latitudinally. The possible effects of seasonal variation can, of course, be eliminated in many isozyme studies. This is accomplished by collecting seed from the population samples and growing the plants under uniform environmental conditions. Many perennial species (i.e., *Juniperus*), however, are not easily grown from seed; yet evaluation of their isozyme variation patterns might contribute to the understanding of the biological significance of these molecular polymorphisms.

Most studies dealing with environmentally induced variation have been concerned with the physiological significance of these changes. The variation in isozyme patterns associated with growth and development in higher plants has been evaluated by using several approaches. Thomas and Neucere (1974) studied changes in peroxidases in peanut seeds and organs of various aged plants. They found a high similarity between banding patterns of different organs, but several qualitative and quantitative ontogenetic differences were detected. Changes in isozyme banding patterns during the growth and development of maize have been studied extensively for several enzyme systems (Brewbaker and Hasegawa, 1975; Scandalios, 1974, 1975). In a detailed study of *Pinus attenuata*, Conkle (1971b) examined changes in alcohol dehydrogenase, leu-

*Present address: Dr. Walter Kelley, Biology Department
Mesa College
Grand Junction, Colorado 81501

cine aminopeptidase, esterase, peroxidase and catalase isozymes during developmental stages. These stages ranged from dry seed to the point where cotyledons were horizontal and the epicotyl was visible (small seedlings). Considerable changes were noted for all enzyme systems with both developmental stage and tissue type (embryo, top, root).

DeJong (1973) investigated the effect of environmental conditions on isozyme banding patterns in tobacco. He found that alternating a single environmental variable produced a different isozyme profile. Similar results were obtained in three *Citrus* species exposed to various photoperiods by Wagner and Upadhya (1968). A series of studies to evaluate the influence of environmental factors upon the esterases of *Pinus silvestris* have been conducted (Rasmuson and Rudin, 1971; Rudin et al., 1974).

Investigations of variation in isozymes over an extended period in perennial plants growing under natural conditions have not been conducted. This study was undertaken to investigate the nature of isozymes in *Juniperus scopulorum* Sarg. during growth under natural environmental conditions over a 12 month period. The peroxidases, esterases, and α-terpineol dehydrogenases were evaluated monthly to detect any seasonal variation which might occur and in order to determine the significance of seasonal variation of isozymes for the use by systematists.

MATERIALS AND METHODS—Foliage samples of four *J. scopulorum* trees were collected on the 15th of each month starting on August 15, 1975 and ending on August 15, 1976. The four trees were growing on the Colorado State University campus and voucher specimens (with exact locations) are on file in the Herbarium of Colorado State University (CS:Walter A. Kelley, 06241976-1 through 06241976-4). In order to evaluate seasonal isozyme variation and its relationship to systematic studies, the foliage samples were intentionally taken using normal field sampling techniques (Adams, 1970). *Juniperus* species, being evergreen, contain leaves which are functional for several years. The current year's growth represents only a small portion of the total green foliage. Samples consisted of fresh foliage from four branches, 10–15 cm long, representing several years growth. Samples were taken at 1.5 m height from four sides of each tree. Trees were mature (20–30 years old).

Enzymes were extracted within 2 h of sample collection by using the extraction protocol developed by Kelley and Adams (1977a).

Extraction buffer—The extraction buffer developed for use with Juniper was a 0.10 M tris-maleate buffer pH 7.00 containing: 0.20 M sodium tetraborate, 0.25 M sodium ascorbate, 0.02 M sodium meta-bisulfite, 0.02 M sodium diethyldithiocarbamate (DIECA), 0.01 M germanium dioxide, and 10% (v/v) dimethylsulfoxide (DMSO). The buffer was prepared by dissolving the germanium dioxide in boiling water; this solution was allowed to cool to 25 C, then the other components were added and the pH adjusted.

Extraction procedure—Ten grams of polyvinylpolypyrrolidone (PVPP) powder were mixed with 50 ml of extraction buffer and allowed to hydrate for 24 h at 6 C. Paste was obtained after hydration of buffer/PVPP. Ten grams of Juniper foliage were placed in a cold mortar with 5 g of alumina and ground with liquid nitrogen until a a fine green powder was obtained. This powder was then added to the buffer/PVPP paste, mixed, and allowed to warm at room temperature for 30 min. The Juniper-buffer/PVPP paste was then expressed through nylon. The liquid obtained was centrifuged at 400 *g* for 10 min at 6 C. The supernatant was retained and mixed with an equal volume of n-butanol in a separatory funnel. A colloidal suspension was obtained which was centrifuged at 400 *g* for 10 min at 6 C. Following centrifugation a three phased suspension was obtained composed of an upper butanol layer, a middle semi-solid layer, and a lower water layer. The water layer was removed and extracted with an equal volume of ethyl ether. The water layer was removed and concentrated by dialysis in powdered sugar for 48 h at 6 C. Following dialysis all samples were stored at −20 C until electrophoresis.

Electrophoresis was preformed within 96 h from extraction, on 4½%, 6%, 8% anodic polyacarylamide gels. Gel preparation, electrophoresis, and staining were conducted by the techniques of Kelley and Adams (1977a). Gels were stained for peroxidases, esterases, and α-terpineol dehydrogenases.

Following staining, Rm values were calculated, data sheets scored, and photographic records made of all gels. After all monthly runs were completed, the bands for the three enzyme systems were numbered. Band numbers were assigned starting with the band nearest the origin (smallest Rm) and ending with the band nearest the anode (largest Rm).

RESULTS—Patterns of variation in isozymes for *J. scopulorum* are shown in Fig. 1 (peroxidases) and Fig. 2 (esterases). The four *J. scopulorum* trees sampled had one band for α-terpineol (alcohol) dehydrogenase (Rm = 0.22) which did not appear to vary during the year.

No qualitative differences were detected in peroxidases (Fig. 1) banding patterns during the 12 month evaluation period. As can be seen in Fig. 1, bands 1, 2, 3, and 4 showed identical

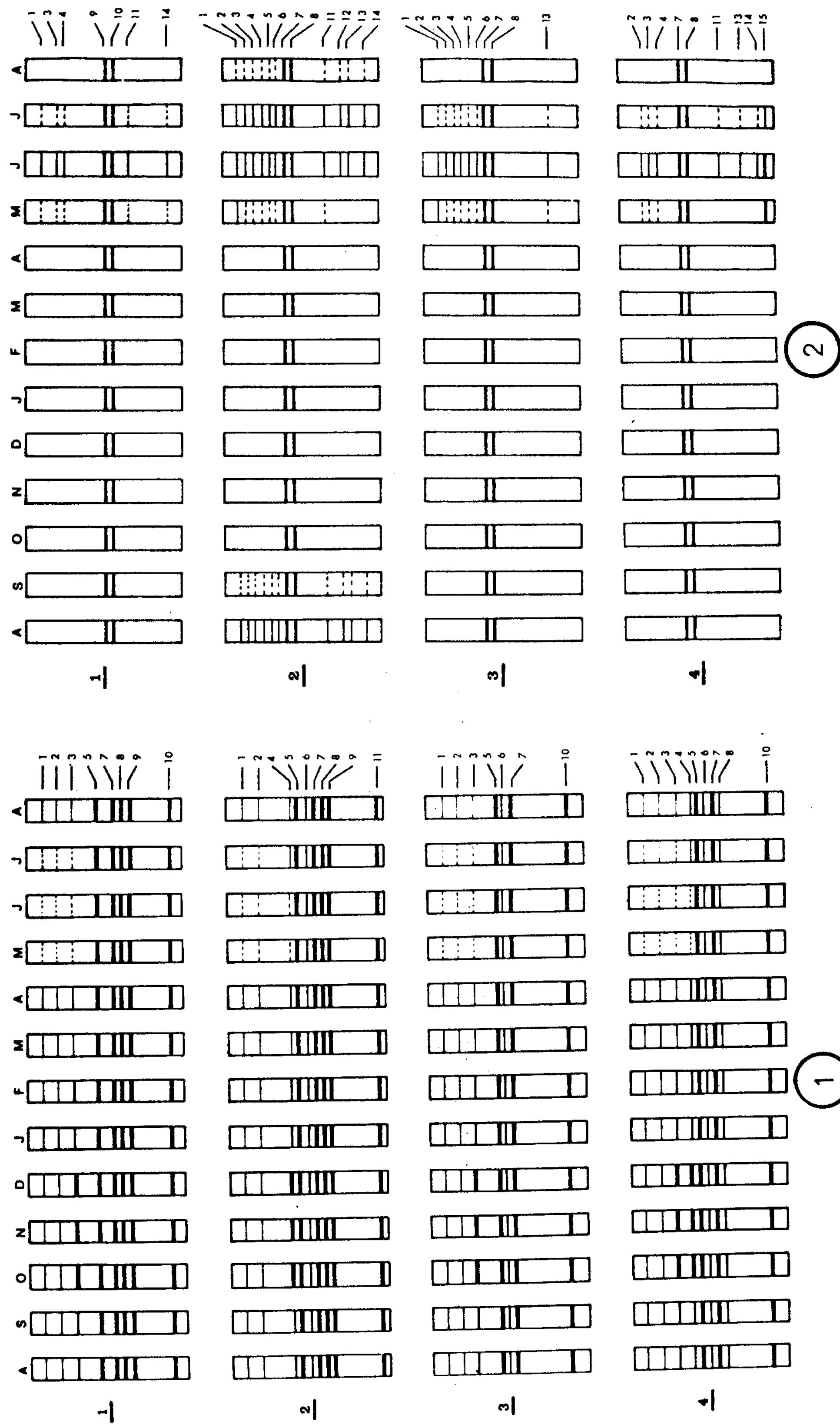

Fig. 1, 2. Zymograms of four *J. scopulorum* trees for peroxidases (Fig. 1) and esterases (Fig. 2). Tree numbers are on left of each figure. These figures represent seasonal variation for each tree starting August 15, 1975 (left) and ending August 15, 1976 (right). Months are noted by letters above samples.

quantitative variation patterns in all trees during the sampling period. Peroxidase bands 3 and 4 were broad and dark-staining from October through December. The staining intensity was lighter from January through April, and in May, June, and July these bands were only barely detectable. This seasonal variation pattern was identical in all trees having bands 3 and/or 4. Isoperoxidase bands 1 and 2 showed uniform staining from August through April, with a reduction in staining intensity in May, June, and July. All other isoperoxidase banding patterns remained constant.

Esterase zymograms (Fig. 2) show qualitative seasonal differences in banding patterns. Esterase bands 7 and 8 (trees 2, 3, and 4) and bands 9 and 10 (tree 1) are found throughout the sampling period; these bands will be referred to as the constant esterases (C-esterases). From August through April all trees except tree 2 had two C-esterases only. In August and September tree 2 had two C-esterases plus ten additional isoesterases. All four trees had only two C-esterases from October through April. In May, June, and July all trees had two C-esterases plus additional esterase bands. The esterases which vary seasonally will be referred to as variable esterases (V-esterases).

The C-esterases were always broad, dark staining bands and the V-esterases except band 15 (tree 4) were always narrow, lighter staining bands. Variable esterase 15 was a broad, dark staining band. All trees showed esterase patterns in May, June, and July which contained a combination of C-esterases and V-esterases. None of the trees showed V-esterases banding from October through April.

Discussion—Peroxidases showed very little seasonal variation other than the quantitative variation detected in bands 1, 2, 3, and 4. Especially pronounced was the apparent increase in production (as noted by darker and broader staining) in bands 3 and 4 in the late fall months. This was followed by a decrease in production of these isozymes in winter and spring. In the late spring these two bands are barely detectable.

Little is known about the precise physiological function of peroxidases in higher plants. Several functions have been attributed to this enzyme. Peroxidases are believed to be associated with cell wall lignification (Freudenberg, 1959; Young and Steelink, 1973), indolacetic acid (IAA) oxidation (Galston, Bonner, and Baker, 1953), the physiology of seed germination (Hendricks and Taylorson, 1975), and the scavenging of H_2O_2 (Fridovich, 1975; Halliwell, 1974).

One can speculate that these two isoperoxidases (bands 3 and 4) which are produced in larger quantities in the late fall are associated with cell wall lignification which may occur at this time. During the spring and summer most cell growth takes place in these trees (Powell and Adams, 1973). Once this cellular growth has ceased, cell wall lignification occurs in many newly formed cells. Perhaps genes are turned on to produce isoperoxidases associated with lignification at the cessation of the growth phase and before winter dormancy has occurred. These genes may be the ones coding for isoperoxidases 3 and 4 in *J. scopulorum*. It is also possible that these two peroxidases (and bands 1 and 2 as well) which show increased production in the late fall are in some way associated with winter hardiness in *J. scopulorum* since their increase in production occurs prior to the winter months and is negligible in the spring and summer.

The other isoperoxidases (excluding bands 1, 2, 3, and 4) are detected in relatively the same amounts throughout the year and may be required for normal cellular function throughout the life of these trees.

It should be noted, based on this investigation of seasonal variation in peroxidases of *J. scopulorum*, that all bands were detectable throughout the 12 month sampling period.

Esterases show a seasonal variation pattern quite different from that seen in the peroxidases. Considerable qualitative seasonal differences were found with most bands being present in the spring and on into the summer. The V-esterases were detected in the late spring in all trees and were found in tree 2 in the late summer (August, 1975, 1976; September, 1975). All trees have a similar pattern of production of these V-esterases with tree 2 apparently being out of synchrony with respect to cessation of their production. For esterases, the most consistent banding (although fewest number of bands) was detected in the late fall and winter when only C-esterases were seen.

The esterases are a complex and heterogeneous group of enzymes, with the natural substrates and in vivo function of most of the esterases in higher plants unknown (Scandalios, 1969). Thus one cannot even speculate upon the biological significance of the increase in production of the V-esterases during the spring and summer. In previous studies (Kelley, 1976; Kelley and Adams, 1977a) it was shown in *J. scopulorum* that the V-esterases are butanol sensitive and therefore possibly are β-esterases. These studies also showed the C-esterases to be insensitive to butanol inhibition and thus are putative α-esterases. The appearance of seasonal variation in production of V-esterases (β-esterases) and constant production of C-esterases (α-esterases) may reflect differing physiological roles for these two classes of esterases in *J. scopulorum*.

Studies of seasonal variation of terpenes in *Juniperus* have shown that the production of these compounds varies seasonally and that the most stable periods are in the fall and winter months

(Adams, 1970; Powell and Adams, 1973). It is not surprising then to find seasonal variation in isozymes in *J. scopulorum* because enzymes are responsible, via cellular gene regulation, for the seasonal variation in production of most cellular constituents. The most stable periods for isozyme production also appear to be the fall and winter. These findings and those for terpenoids are in keeping with the idea of reduction in plant growth during the fall and winter (a dormant season), which leads to stability in enzymatic and chemical composition in conifers.

The amount and nature of seasonal variation in the isoesterases of *J. scopulorum* should stand as a warning to those conducting systematic studies. This is especially true when foliage samples taken from natural populations are the source of enzyme extracts. A systematist wishing to evaluate the nature of isoesterase polymorphisms in this species would obtain some perplexing and erroneous results if some of the population sampled were dormant while others had broken dormancy. The patterns of isoesterase polymorphisms would be greatly distorted by natural seasonal variation which occurs in this species. A systematist who investigates chemical characters under physiological control must be certain that his populations are phenologically similar.

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