Phenylalanine ammonia lyase as a precursory enzyme of legume stem lignification

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[1]*Biology Department, University of Central Oklahoma, 100 North University Drive, Edmond, Oklahoma 73034, USA;* [2]*United States Department of Agriculture — Agricultural Research Service, 1577 Agronomy Hall, Iowa State University, Ames, Iowa 50011, USA; and* [3]*Department of Agronomy, Iowa State University, Ames, Iowa 50011, USA.* *Joint contribution of Iowa State University and Field Crops Research Unit and U.S. Dairy Forage Research Center of USDA Agriculture Research Service Journal Paper no. J-14202 of the Iowa Agriculture and Home Economics Experiment Station, Ames; Project 2709. Received 7 April 1994, accepted 19 September 1994.*

Bidlack, J. E., Buxton, D. R., Shibles, R. M. and Anderson, I. C. 1995. **Phenylalanine ammonia lyase as a precursory enzyme of legume stem lignification.** Can. J. Plant Sci. 75: 135–140. In some instances, lignin content may not be significantly correlated with phenylalanine ammonia lyase (PAL) activity because: (1) PAL is not committed exclusively to lignin, and (2) the time of maximum PAL activity may not coincide with maximum lignin deposition. This study evaluates correlations and timing of PAL activity and lignin deposition during legume stem maturation. Three forage legumes, alfalfa (*Medicago sativa* L.), birdsfoot trefoil (*Lotus corniculatus* L.), and red clover (*Trifolium pratense* L.), were established, and basal stem regrowth was sampled, biweekly, for 10 wk, for dry weight (DW), cell wall (CW), lignin, and PAL analyses. Nonlinear regression of lignin content by the Gompertz function indicated that lignin increased sigmoidally, and PAL activity by the third-order quadratic demonstrated rapid initial increases in activity, followed by decreases, as a function of regrowth days. First derivative of the Gompertz function demonstrated that changes in lignin deposition closely resembled changes in PAL activity. Among species, peak deposition of DW and CW content occurred 3–11 d prior to maximum lignin deposition. Time of maximum PAL activity occurred 8 d prior to maximum lignin deposition in birdsfoot trefoil and red clover and 3 days after maximum lignin deposition in alfalfa. Across species, lignin content was not positively correlated with PAL activity on a protein basis. However, lignin deposition was positively correlated with PAL per unit protein ($r = 0.76$, $P < 0.05$) and lignin content was positively correlated with PAL on a per plant basis ($r = 0.60$, $P < 0.05$). These results indicate that the activity of PAL is related to lignin deposition in a cause-and-effect relationship.

Key words: Alfalfa, birdsfoot trefoil, cell wall, lignin, phenylalanine, ammonia lyase, red clover

Bidlack, J. E., Buxton, D. R., Shibles, R. M. et Anderson, I. C. 1995. **Role de la phénylalanine, ammoniac-lyase comme précurseur de la lignification de la tige chez les légumineuses.** Can. J. Plant Sci. 75: 135–140. Dans certains cas, le contenu en lignine n'est pas significativement corrélé avec l'activité de la phénylalanine ammoniac-lyase (PAL) et cela pour les raisons suivantes: 1) l'activité de PAL n'est pas exclusivement limitée à la synthèse de la lignine et 2) le temps de l'activité maximum de la PAL ne coïncide pas nécessairement avec celui de l'accumulation de lignine maximum. L'objet de nos travaux était d'évaluer les corrélations et le degré de synchronisme de l'activité PAL et de la formation de la lignine au cours de la maturation de la tige des légumineuses. L'expérience portait sur trois légumineuses fourragères: luzerne (*Medicago sativa* L.), lotier corniculé (*Lotus corniculatus* L.) et trèfle rouge (*Trifolium pratense* L.). Toutes les deux semaines pendant dix semaines, on prélevait des échantillons de la partie inférieure des repousses de la tige pour les analyses du poids sec (PS), de parois cellulaires (PC), de la teneur en lignine et de l'activité de PAL. Une régression non linéaire du contenu en lignine obtenue par la fonction de Gompertz, et de l'activité de la PAL obtenue par équation quadratique du troisième ordre révèle que le contenu en lignine augmentait en fonction sigmoïdale et que PAL démontrait des accroissements initiaux rapides, suivis d'une diminution de l'activité en fonction du nombre de jours de repousse. La première dérivée de la fonction de Gompertz révèle en outre que l'évolution de l'accumulation de lignine concordait étroitement avec celle de l'activité PAL. Parmi les différentes espèces, le pic d'accumulation de poids sec et de formation de parois cellulaires s'observaient de 3 à 11 jours avant la formation maximum de lignine. Le temps de l'activité PAL maximum survenait 8 jours avant le dépôt maximum de lignine chez le lotier et chez le trèfle rouge et 3 jours après chez la luzerne. Toutes espèces confondues, la teneur en lignine n'était pas positivement corrélée avec l'activité PAL exprimée en fonction de la teneur en protéines, mais elle l'était quand l'activité PAL était exprimée par gramme de protéines (R–0,76 $P < 0,05$). De plus, calculée par plante individuelle, la teneur en lignine était positivement corrélée avec PAL ($r = 0,60$ $P < 0,05$). Il ressort de ces observations que l'activité de la PAL est reliée avec l'accumulation de la lignine, dans une relation de cause à effet.

Mots clés: Luzerne, lotier corniculé, parois cellulaires, lignine, phénylalanine ammoniac-lyase, trèfle rouge

Digestibility and potential intake of forages by ruminants is associated with CW and CW component concentration (Van Soest and Robertson 1980). Of CW components, lignin is usually the factor correlated with lowering digestibility of maturing forage tissues (Jung and Deety 1993). Previous studies revealed much information about the quantity

Abbreviations: **CAD**, cinnamyl alcohol dehydrogenase; **CW**, cell wall; **DW**, dry weight; **PAL**, phenylalanine ammonia lyase

(Buxton and Hornstein 1986) and quality (Buxton and Russell 1988) of lignin in relation to digestibility. Bidlack and Buxton (1992) showed that maximum lignin deposition in legume stems occurs within the first 35 d of regrowth of aftermath forage growth. Whether or not this lignin deposition can be directly attributed to enzymes involved in lignification has yet to be determined.

Lignin biosynthesis begins with the conversion of phenylalanine to cinnamic acid (Hahlbrock and Grisebach 1979; Lewis and Yamamoto 1990). The enzyme catalyzing this reaction, PAL, has been investigated in several crop species (Kishor 1989; Dalkin et al. 1990) and its positive effect on lignification has been established (Grand et al. 1982; De Jaegher et al. 1985; Guerra et al. 1985). Recent work (Morrison and Buxton 1993; Morrison et al. 1994), however, reveals no significant correlation between PAL activity and increase in lignin concentration of maize (*Zea mays* L.) stalks during maturation. Lack of significant correlation was attributable to the time lag between maximum PAL activity and lignification in maize. Activity of PAL on a per plant basis (PAL total units) may be more closely related to lignin deposition because the amount, as well as the activity of the PAL enzyme, is attributable to the quantity of lignin accumulated.

Relationships between PAL activity and lignin deposition in other plant species may provide needed information for manipulation of plant mechanical strength, protection against pests, and improved digestion by ruminant animals. Legumes deposit higher concentrations of lignin in their stems than do most grass species, and legumes deposit lignin earlier relative to other CW components than do grasses (Buxton and Russell 1988; Bidlack and Buxton 1992). Hence, the temporal relationships between PAL activity and lignin deposition may differ between legumes and grasses.

The goal of this investigation was to determine the temporal relationship between PAL activity and lignin deposition in three cool-season legume species. Better understanding of the temporal relationships between enzyme activity and lignification will improve current conceptualization of secondary CW development and provide a foundation for targeted alteration of the chemical composition of secondary CWs. The objectives of this study were to (1) determine the relative timing of DW, CW, and lignin deposition in relation to changes in PAL activity of legume stems, and (2) determine whether significant relationships exist between PAL activity, on a protein and per plant basis, and lignin deposition within and across cool-season legume species.

MATERIALS AND METHODS

The three legume species studied were Arrow alfalfa (*Medicago sativa* L.), Viking birdsfoot trefoil (*Lotus corniculatus* L.), and Arlington red clover (*Trifolium pratense* L). These species were planted during spring 1987 in a greenhouse in 25-cm-diameter pots with a capacity of 3.8 L. Plants were thinned to three seedlings per pot after establishment and were cut once every 10 wk until the beginning of the sampling period. Plants were watered as needed and fertilized once a week with Hoagland solution. Each pot contained a 1:2:2:4 mixture (by volume) of sand, Webster silty clay loam soil (fine loamy, mixed, mesic Typic Haplaquolls), peat, and perlite.

Pots were arranged in a randomized complete-block design of the three species in four replicates. In each pot, an average of the three legumes was used to express values on a per plant basis. A split-plot arrangement was used, with species as the whole plot and sample age as the subplot. Greenhouse temperatures during all sampling periods ranged from 21 to 34°C and high-pressure sodium lamps supplemented sunlight to provide a 14-h photoperiod.

Plant Sampling

All forage was cut at 3–5 cm above soil level in September 1988 and sampled during October through December 1988 at 14, 28, 42, 56 and 70 d after regrowth. The basal 10 cm of harvested stem material was weighed and subdivided for fiber and enzyme analyses. Basal stem tissue of legumes was used in this investigation to attain uniformity of sampling and take advantage of the large variation that results during maturation of these tissues (Buxton and Russell 1988). Five grams of fresh material was quick frozen in liquid N_2 for PAL analysis. Remaining stem material was put into individual paper bags and dried at 55°C for 48 h for fiber analysis.

Enzyme Extraction and Assay

Acetone powders of the frozen stem samples were prepared to avoid problems that may be encountered in enol tautomer-borate complexes associated with direct aqueous extracts (Erez 1973). Approximately 5.0 g of frozen stem material was homogenized in acetone (−78°C, 200 mL) with a blender for 2 min. The mixture was transferred with an additional 50 mL of cold acetone to a 500-mL square jar and ground with a tissue homogenizer for 3 min. The resulting acetone powder was collected by Büchner filtration through Whatman No. 54 filter paper and rinsed with 50 mL of cold acetone. After air-drying for 1 h, the powder was stored in an ultra-low freezer at −100°C.

Complete replicates were removed from the freezer and 25 mg of acetone powder from individual samples was mixed with 25 mL of 100 mM borate buffer, pH 8.7, at 0 to 2°C. Suspensions were shaken at least once every 5 min for 1 h and then filtered through Whatman No. 54 filter paper to give a clear, crude enzyme extract. These extracts contained PAL associated with the endoplasmic reticulum, which is known to be involved with lignin biosynthesis (Wagner and Hrazdina 1984). Duplicate protein concentrations, in acetone powder, were determined (Bradford 1976) and multiplied by the acetone powder yield per plant to obtain protein on a per plant basis.

The PAL enzyme was assayed spectrophotometrically from a mixture of 1.5 mL of extract and 4.5 mL of 6.68 mM phenylalanine in 100 mM borate buffer (pH 8.7) over a period of 1 h at 30°C in duplicate. Concentration of phenylalanine in the assay mixture was 5.01 mM. Enzyme activity according to Abell and Shen (1988) was obtained by measuring production of cinnamic acid at 290 nm using an extinction coefficient of 9000 M^{-1} cm^{-1}. Values were corrected by subtracting activity of a blank lacking

enzyme extract. One unit of PAL activity was expressed as the amount of cinnamic acid produced in μmol h^{-1}. These activities, originally expressed on a per gram protein basis, were multiplied by the protein yield (in acetone powder) of each plant to express PAL on a units per plant basis.

Fiber Analysis

Dried samples were weighed and ground to pass through a 1-mm screen of a Udy Mill. Sequential fiber analysis, outlined by Van Soest and Robertson (1980), was used to determine the amount of lignin and other CW components in the ground stem samples. Lignin was calculated as the acid detergent lignin residue isolated from a 72% H_2SO_4 extraction minus the ash weight.

Statistical Analysis and Graphical Representations

Analysis of variance (ANOVA) was performed by the general linear model procedure in SAS (SAS PROC GLM; SAS Institute, Inc. 1985). The split-plot design was implemented to obtain ANOVAs that provided a pooled error term for species × age interactions. Simple ANOVAs were used to determine differences in timing among components within species or differences in timing among species within components. Least significant differences at the 0.05 level in ANOVAs were calculated from the standard error of the mean. Nonlinear regression R^2 values for the Gompertz function and the third-order quadratic were calculated by dividing the residual sum of squares (from SAS PROC NLIN) by the corrected total sum of squares and subtracting from one (Hattendorf et al. 1988).

Dry weight, CW, and lignin content were expressed on a per plant basis for relative comparison. Graphical representations of these measurements as a function of regrowth days were performed by fitting data to the Gompertz function

$$Y = a \times \exp\,[(-b) \times \exp\,(-ct)]$$

where Y is component measured, a is maximum value of component, b is relative growth rate as affected by t, c is estimated constant, and t is time in days (Hunt 1982). Values for b and c were estimated by computer with the nonlinear regression procedure in SAS (SAS PROC NLIN; SAS Institute, Inc. 1985), and values for a were entered as the highest number from data within each species. Graphs for DW deposition, CW deposition and lignin deposition, were constructed from the first derivative of the Gompertz function with respect to regrowth days,

$$dY/dt = abc \times \exp\,(-ct) \times \exp\,[(-b) \times \exp\,(-ct)].$$

This function optimizes growth response curves and has been used in other biological studies (Pegelow et al. 1977; Hattendorf et al. 1988; Bidlack and Buxton 1992).

Activity of PAL on a protein basis was represented by fitting data to the third-order quadratic

$$Y = at^3 + bt^2 + ct + d$$

where y is PAL activity, a, b, c, and d are coefficients estimated by SAS PROC NLIN (SAS Institute, Inc. 1985), and t is time in days. This equation was used because it provided a good demonstration of drastic increases and decreases in enzyme activity.

Times of maximum DW, CW and lignin deposition were determined from average values over replicates by setting the second derivative of the Gompertz function, which has been corrected from Bidlack and Buxton (1992)

$$d^2Y/dt^2 = ab^2c^2 \times \exp\,[\exp\,(-ct)]^2 \times \exp\,[(-b) \times \exp\,(-ct)] - abc^2 \times \exp\,(-ct) \times \exp\,[(-b) \times \exp\,(-ct)]$$

equal to 0 and solving for t. Time of maximum PAL activity was determined from average values over replicates by setting the first derivative of the third-order quadratic,

$$dY/dt = 3at^2 + 2bt + c$$

equal to 0 and solving for t.

Linear regressions for lignin content vs. PAL activity were performed from means of the four replications, excluding the point 0,0.

RESULTS AND DISCUSSION

Lignin content in alfalfa ($Y = 126 \times \exp\,[(-3.88) \times \exp\,(-0.087 \times t)]$) birdsfoot trefoil ($Y = 134 \times \exp\,[(-3.46) \times \exp\,(-0.043 \times t)]$), and red clover ($Y = 37.1 \times \exp\,[(-3.14) \times \exp(-0.044 \times t)]$) increased significantly as a function of regrowth days (top panel of Fig. 1). Use of the Gompertz function to express increases in lignin content of alfalfa ($R^2 = 0.98$), birdsfoot trefoil ($R^2 = 0.92$) and red clover ($R^2 = 0.90$) indicated that at least 90% of the variation could be explained by this equation as a function of plant age. Good fit of lignin data is attributable to sigmoidal increases optimized by the Gompertz function (Hunt 1982). Other studies (Bidlack and Buxton 1992) have shown that amount of DW, CW, hemicellulose, and cellulose increase in a sigmoidal fashion with appropriate lag, log, and leveling-off phases.

While lignin content increased sigmoidally, PAL on a protein basis demonstrated initial increases followed by a decrease or lateralization of activity (bottom panel of Fig. 1). The peak of PAL activity shown in the bottom panel of Fig. 1 closely corresponds with the logarithmic increase in lignin content shown in the top panel of Fig. 1. Increases in PAL activity during the first 20 d of regrowth and decreases in PAL activity beyond 30 d of regrowth were significant in all species. Increases and decreases in PAL activity coincided with the lag and leveling-off phases of lignin accumulation. Skewed, bell-shaped responses of PAL activity, as a function of regrowth days, were well fit by the third-order quadratic equation for alfalfa ($R^2 = 0.91$), birdsfoot trefoil ($R^2 = 0.83$), and red clover ($R^2 = 0.99$). Similarities among the first three coefficients for alfalfa ($Y = 0.00328t^3 - 0.406t^2 + 13.0t + 6.22$), birdsfoot trefoil ($Y = 0.00272t^3 - 0.335t^2 + 10.6t + 7.44$), and red clover

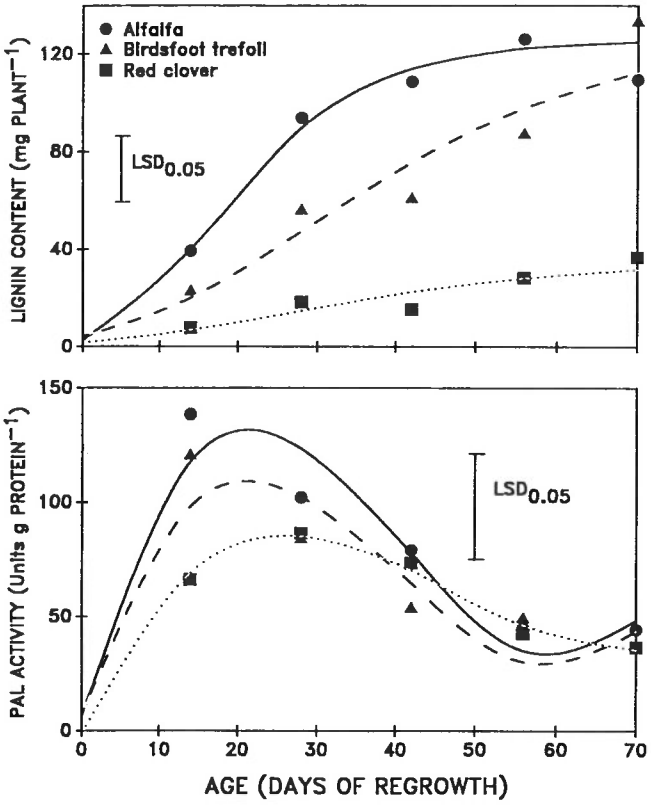

Fig. 1. Lignin content of basal stem regrowth from alfalfa, birdsfoot trefoil and red clover with R^2 values of 0.98, 0.92 and 0.90, respectively; and PAL activity from acetone powders of alfalfa, birdsfoot trefoil and red clover with R^2 values of 0.91, 0.83 and 0.99, respectively, as a function of regrowth days. Each point represents the mean of four values.

($Y = 0.00135t^3 - 0.194t^2 + 7.44t - 0.994$) indicated that the shapes of the curves were similar. The fourth coefficient (Y-intercept) implied that alfalfa, birdsfoot trefoil, and red clover demonstrated detectable PAL activity at 6.22, 7.44 and 0 d, respectively, after regrowth. Dramatic increases in enzyme activity during early growth, followed by a leveling off or decrease in PAL have been encountered in rice (*Oryza sativa* L.) callus cultures (Kishor 1989), wheat (*Triticum aestivum* L.) grain development (McCallum and Walker 1990), and maize stalk maturation (Morrison and Buxton 1993; Morrison et al. 1994).

Changes in lignin deposition and PAL activity were more obvious in alfalfa than in birdsfoot trefoil and red clover, suggesting that CW deposition and lignification were most rapid in alfalfa. Faster lignification in alfalfa would be expected, since previous studies of these same plants have shown that alfalfa achieved reproductive maturity faster (Bidlack and Buxton 1992) than in other legumes. In birdsfoot trefoil and red clover, curves demonstrated by PAL activity (Fig. 1) were much sharper than those demonstrated by DW, CW and lignin deposition (Bidlack and Buxton 1992). Thus, in these species, PAL induction and cessation may have been more sharply regulated than the initial and final steps of CW synthesis.

Table 1. Estimated times of maximum dry weight (DW) and cell wall (CW) component deposition on a per plant basis and maximum phenylalanine ammonia lyase (PAL) activity on a per-protein basis of stem base regrowth harvested from greenhouse-grown legumes[z]

Measurement	Species			$LSD_{0.05}$
	Alfalfa	Birdsfoot trefoil	Red clover	
	(d)			
Stem base DW	12.7	27.2	27.7	9.6
CW	14.9	28.2	23.0	8.3
PAL activity	20.3	21.5	25.3	3.7
Lignin	17.4	30.0	34.0	8.9
$LSD_{0.05}$	1.6	3.8	9.7	

[z] Times of maximum DW and CW deposition were calculated by setting the second derivative of the Gompertz function equal to 0 and solving for t; time of maximum PAL activity was calculated by setting the second-order quadratic equal to 0 and solving for t.

Comparison of peaks among DW, CW, lignin deposition (Bidlack and Buxton 1992) and PAL activity (lower panel of Fig. 1) revealed differences in time of maximum component deposition and PAL activity among and within species. Peak deposition of DW, CW and lignin in red clover lagged behind deposition of these components in alfalfa. Time of maximum PAL activity in red clover also lagged behind time of maximum PAL activity in alfalfa and birdsfoot trefoil. Other studies have shown that DW, CW and CW component accumulation in red clover lags behind the same measurements in alfalfa and birdsfoot trefoil (Buxton and Hornstein 1986). In birdsfoot trefoil and red clover, time of maximum PAL activity preceded the time of maximum lignin deposition. Derivatives of equations for these lines were exploited to reveal a tentative chain of events leading to lignification in stem bases of cool-season legumes.

Significant differences were encountered for times of maximum deposition of DW, CW and lignin, as well as maximum PAL activity among species and for measurements within species (Table 1). In birdsfoot trefoil and red clover, time of maximum PAL activity was the same as or preceded the time of maximum lignin deposition. Maximum PAL activity was achieved later than maximum lignin deposition in alfalfa. Optimization of PAL activity after maximum lignin deposition in alfalfa may be due to activation of the enzyme by pathogenic infection (Dalkin et al. 1990), light (Guerra et al. 1985) or other environmental stimuli known to increase PAL more than 100-fold (Hahlbrock and Grisebach 1979). Since PAL activity precedes flavonoid, anthocyanin, phytoalexin, tannin and other phenylpropanoids, in addition to lignin, it is possible that maximum activity of PAL does not always precede maximum lignin deposition. Even though Table 1 shows that maximum lignin deposition followed maximum PAL activity in alfalfa, the difference was less than 3 d.

Maximum deposition of DW, CW and lignin occurred significantly earlier in alfalfa than in birdsfoot trefoil and red clover (Table 1). Maximum PAL activity occurred significantly earlier in alfalfa and birdsfoot trefoil than in red clover. The lag between maximum PAL activity and maximum lignin deposition was highest in birdsfoot trefoil. Time of maximum PAL activity in birdsfoot trefoil occurred 6–8 d

prior to maximum deposition of all other components measured in this species.

In all species, maximum DW and CW deposition occurred prior to maximum lignin deposition. Maximum PAL activity was usually earlier than maximum lignin deposition. Activation of PAL prior to lignification concurs with results of Morrison and Buxton (1993) and Morrison et al. (1994) and supports the idea that PAL is required for synthesis of phenylpropanoid products during early cell differentiation. Initial increases in PAL activity, coupled with concurrent increases in lignin concentration and cinnamyl alcohol dehydrogenase (CAD) activity, indicate that lignification in maize is indirectly attributable to PAL and directly attributable to CAD activity (Morrison and Buxton 1993; Morrison et al. 1994).

Correlation of PAL activity on a protein basis with lignin content revealed a coefficient of $r = -0.35$ ($P > 0.05$) among species. None of the correlations within species was significant and positive, indicating the activity of PAL at any one moment could not be related to the amount of lignin present in basal stem tissue. The time lag between maximum PAL activity and maximum lignin deposition may partially explain why PAL on a protein basis and lignin on a per plant basis were not positively correlated.

Correlations between PAL activity per gram of protein and lignin deposition were evaluated to determine whether or not PAL per unit protein could explain the amount of lignin being deposited at any one moment. Correlation of PAL activity with lignin deposition demonstrated a coefficient of $r = 0.76$ ($P < 0.05$) among species and correlation coefficients of $r = 0.98$ ($P < 0.05$), $r = 0.56$ ($P > 0.05$), and $r = 0.97$ ($P < 0.05$) for alfalfa, birdsfoot trefoil and red clover, respectively. Positive and significant correlations among these measurements indicated that accumulation of lignin is attributable to PAL activity that occurred at some time prior to, or during, lignification.

When data from all species were combined on a per-plant basis, the correlation between PAL activity and lignin content ($r = 0.60$) was significant at the 0.05 level of probability. Correlations within species were not significant. Greater variation in PAL and lignin across species, which can be attributed to plant age and species differences, enabled significance of the lignin vs. PAL correlation. Induction of PAL through fungal elicitors (Dalkin et al. 1990) and rubbing (De Jaegher et al. 1985) also dramatize variations in PAL activity and provide a foundation for significant correlations between lignin and PAL. Correlations as high as $r = 0.99$ have been reported for lignin concentration vs. PAL activity in wheat as affected by light intensity (Guerra et al. 1985). Even though the lignin vs. PAL correlation may not show up as significant in some species (Morrison and Buxton 1993), it is evident from this and other studies (Grand et al. 1982; De Jaegher et al. 1986; Guerra et al. 1985) that lignification is attributable to the precursory PAL enzyme.

Linear regression of lignin vs. PAL across species indicated that 37% of the variation in lignin could be attributed to the PAL enzyme (Fig. 2). Regression lines of birdsfoot trefoil ($Y = 37.8X - 7.56$; $r^2 = 0.47$) and red clover ($Y = 11.6X - 1.88$; $r^2 = 0.50$) (see dotted lines in Fig. 2)

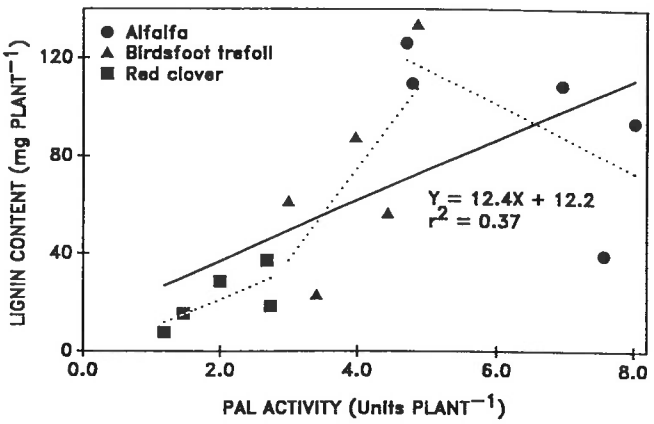

Fig. 2. Relationship between lignin content and PAL activity in stem bases from greenhouse-grown forage legumes as a function of regrowth days. The solid line and equation represent regression across species, and dotted lines indicate regressions within species. Each point represents the mean of four values.

demonstrated that PAL activity was more closely related to lignin content in these species compared to alfalfa ($Y = -13.9X + 184$; $r^2 = 0.42$). The negative y intercepts of birdsfoot trefoil and red clover indicated that PAL activity was detectable prior to quantitation of lignin. These results concur with those of Table 1 whereby maximum PAL activity in birdsfoot trefoil and red clover preceded maximum lignin deposition. Maximum PAL activity in alfalfa may have occurred after maximum lignin deposition because of the aforementioned stimulation by the environment or because quick lignification in alfalfa stems did not allow for accurate estimation of temporal relationships. The multiple roles of PAL in phenylpropanoid metabolism further explain why maximum activity of PAL did not always precede maximum lignin deposition. More information will be needed to show how PAL isozymes and other enzymes committed to lignin affect lignification before scientists can accurately use this knowledge to manipulate plant growth and development.

CONCLUSIONS

These studies and others (Morrison and Buxton 1993; Morrison et al. 1994) indicate that maximum PAL activity precedes or closely parallels cell elongation and the beginning stages of CW development. Activity of PAL may correlate with large changes in lignin deposition as a result of environmental stimuli or differences among species. Within species, activity of enzymes closer to the terminal events of lignification may be more closely related to lignin content. Lack of significant correlations between lignin content and PAL activity within species may be a result of the time lag between maximum PAL activity and maximum lignin deposition. Significant correlations can be acquired between PAL activity and lignin deposition as well as between PAL activity on a per plant basis and lignin content. Future studies should recognize that, even though PAL activity may not be directly correlated with lignin in some species, the catalytic function of PAL is an essential precursor of lignification. Activity of PAL may be highly regulated by subsequent events along the phenylpropanoid pathway.

Abell, C. W., and Shen, R. 1988. Phenylalanine ammonia-lyase from the yeast *Rhodotorula glutinis*. Pages 242–253 *in* S. P. Colowick and N. O. Kaplan, eds. Methods in enzymology. Volume 142. Academic Press, New York, NY.

Bidlack, J. E. and Buxton, D. R. 1992. Content and deposition rates of cellulose, hemicellulose, and lignin during regrowth of forage grasses and legumes. Can J. Plant Sci. **72**: 809–818.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**: 248–254.

Buxton, D. R. and Hornstein, J. S. 1986. Cell-wall concentration and components in stratified canopies of alfalfa, birdsfoot trefoil, and red clover. Crop Sci. **26**: 180–184.

Buxton, D. R. and Russell, J. R. 1988. Lignin constituents and cell wall digestibility of grass and legume stems. Crop Sci. **28**: 553–558.

Dalkin, K., Edwards, R., Edington, B. and Dixon, R. A. 1990. Stress responses in alfalfa (*Medicago sativa* L.). I. Induction of phenylpropanoid biosynthesis and hydrolytic enzymes in elicitor-treated cell suspension cultures. Plant Physiol. **92**: 440–446.

De Jaegher, G. N. Boyer, N. and Gaspar, Th. 1985. Thigomomorphogenesis in *Bryonia dioica*: changes in soluble and wall peroxidases, phenylalanine ammonia-lyase activity, cellulose, lignin content, and monomeric constituents. Plant Growth Regul. **3**: 133–148.

Erez, A. 1973. Possible errors in quantitative determination of phenylalanine ammonia-lyase activity by spectrophotometric methods. Plant Physiol. **51**: 409–411.

Grand, C., Boudet, A. M. and Ranjeva, R. 1982. Natural variations and controlled changes in lignification process. Holzforschung. **36**: 217–223.

Guerra, D., Anderson, A. J. and Salisbury, F. B. 1985. Reduced phenylalanine ammonia-lyase and tyrosine ammonia-lyase activities and lignin synthesis in wheat grown under low pressure sodium lamps. Plant Physiol. **78**: 126–130.

Hahlbrock, K. and Grisebach, H. 1979. Enzymic control in the biosynthesis of lignin and flavonoids. Ann. Rev. Plant Physiol. **30**: 105–130.

Hattendorf, M. J., Carlson, R. E., Halim, R. A. and Buxton, D. R. 1988. Crop water stress index of water-deficit-stressed alfalfa. Agron. J. **80**: 871–875.

Hunt, R. 1982. Plant growth curves: The functional approach to plant growth analysis. Edward Arnold., London, U.K.

Jung, H. G. and D. A. Deety. 1993. Cell wall lignification and degradability. Pages 315–346 *in* H. G. Jung, D. R. Buxton, R. D. Hatfield, and J. Ralph, ed. Forage cell wall structure and digestibility. American Society of Agronomy, Madison, WI.

Kishor, P. B. K. 1989. Activities of phenylalanine- and tyrosine-ammonia lyases and aminotranferases during organogenesis in callus cultures of rice. Plant Cell Physiol. **30**: 25–29.

Lewis, N. G. and Yamamoto, E. 1990. Lignin: Occurrence, biogenesis and biodegradation. Ann. Rev. Plant Physiol. Plant Mol. Biol. **41**: 455–496.

McCallum, J. A. and Walker, J. R. L. 1990. Phenolic biosynthesis during grain development in wheat: changes in phenylalanine ammonia-lyase activity and soluble phenolic content. J. Cereal Sci. **11**: 35–49.

Morrison, T. A. and Buxton, D. R. 1993. Activity of phenylalanine ammonia lyase, tyrosine ammonia lyase, and cinnamyl alcohol dehydrogenase in maize stalk. Crop Sci. **33**: 1264–1268.

Morrison, T. A., Kessler, J. R., Hatfield, R. D. and Buxton, D. R. 1994. Activity of two lignin biosynthesis enzymes during development of a maize internode. J. Sci. Food Agric. **65**: 133–139.

Pegelow, E. J., Taylor, B. B., Horrocks, R. D., Buxton, D. R., Marx, D. B. and Wanjura, D. F. 1977. The Gompertz function as a model for cotton hypocotyl elongation. Agron. J. **69**: 875–878.

SAS Institute, Inc. 1985. SAS user's guide: Statistics. 5th ed. SAS Institute, Cary, NC.

Van Soest, P. J. and Robertson, J. B. 1980. Systems of analysis for evaluating fibrous feeds. Pages 49–60 *in* W. J. Pigden, C. C. Balch, and M. Graham, eds. Proc. Int. Workshop on Standardization Analytical Methodology Feeds, IDRC, Ottawa, ON. 12–14 March 1979. Rep. 134e International Development Research Center, Ottawa, ON.

Wagner, G. J. and Hrazdina, G. 1984. Endoplasmic reticulum as a site of phenylpropanoid and flavonoid metabolism in *Hippeastrum*. Plant Physiol. **74**: 901–906.