Seasonal Changes in Nonstructural Carbohydrates, Protein, and Macronutrients in Roots of Alfalfa, Red Clover, Sweetclover, and Birdsfoot Trefoil

Rong Li, J. J. Volenec,* B. C. Joern, and S. M. Cunningham

ABSTRACT

Root total nonstructural carbohydrate (TNC) has been studied, but little is known about seasonal trends in proteins and other macronutrients in legume roots. Our objectives were to determine how winter hardening and resumption of growth in spring influence (i) concentrations of sugars and starch, and activities of amylases; and (ii) protein concentration and composition, and abundance of N, P, and K. Alfalfa (Medicago sativa L.), red clover (Trifolium pratense L.), birdsfoot trefoil (Lotus corniculatus L.), and biennial sweetclover (Melilotus officinalis L.) were seeded in rows in the field in May and roots were sampled at approximately monthly intervals in autumn and biweekly in spring. All species accumulated high root TNC and starch concentrations in autumn. Starch and TNC concentrations declined throughout winter, but increased in roots of alfalfa and red clover in May. Starch accumulation and degradation patterns in spring were correlated with root endoamylase activity. Root sugar concentrations increased threefold from October to November. Root N and soluble protein concentrations increased 50% between September and December, and declined between March and May. Specific proteins accumulated to high concentrations in alfalfa roots during autumn. Root P increased 50% between October and April and was associated with an increase in root phytate. Proteins, N, P, and phytate accumulated during winter hardening in autumn in roots of these forage legume species that, in conjunction with TNC, may supply nutrients to regrowing shoots in spring.

Accumulation of starch and sugar in roots is often associated with defoliation tolerance and persistence of perennial forage legumes (Graber et al., 1927; Grandfield, 1943; Smith, 1962, 1964). Roots exhibit a cyclic pattern of TNC depletion after shoot removal followed by a rapid increase in root TNC during the third and fourth weeks of shoot regrowth. In addition, large changes in root starch and sugar concentrations occur as plants winter harden in autumn and when shoot growth resumes in spring. The conversion of root starch to soluble sugars is thought to enhance plant tolerance to low temperature and other stresses associated with winter (Bula et al., 1956; Ruelke and Smith, 1956), but the biochemical basis for starch degradation in legume roots remains unknown. In addition, recent results indicate that high root starch concentrations are not required for defoliation tolerance and winter survival of alfalfa (Boyce and Volenec, 1992b). This suggests that other root constituents also may influence defoliation tolerance and winter hardiness.

More recent studies suggest that N-containing compounds in roots, including proteins and amino acids, are used as an N source for shoot regrowth after defoliation (Cralle and Heichel, 1981; Phillips et al., 1983; Davidson et al., 1990; Hendershot and Volenec, 1993b; Kim et al., 1991, 1993; Ourry et al., 1994) and in spring when shoot growth resumes (Volenec et al., 1991; Hendershot and Volenec, 1993a). Use of root N pools for shoot growth may occur because defoliation dramatically reduces nodule nitrogenase activity (Vance et al., 1979) and root mineral N uptake (Kim et al., 1993). In alfalfa, three polypeptides with molecular masses of 32, 19, and 15 kD preferentially accumulate in alfalfa roots in autumn and diminish in spring and after defoliation when crown meristems are reactivated. These polypeptides are present in great abundance over others in root extracts (Volenec et al., 1991; Hendershot and Volenec, 1993a, b). Alfalfa roots contain very high exoamylase concentrations (Boyce and Volenec, 1992a), that exhibit patterns of activity similar to that of the 32-, 19-, and 15-kD polypeptides described above (Volenec et al., 1991). These patterns of protein accumulation in and depletion from roots are consistent with functions assigned to VSPs (Cyr and Bewley, 1990). It is unknown whether roots of other forage legume species accumulate VSPs or exoamylases similar to alfalfa. Nor do we know if concentrations of other macronutrients in roots exhibit cyclic patterns of accumulation and depletion as observed for C and N.

Our objectives were to determine how winter hardening and resumption of growth in spring influence (i) concentrations of sugars and starch, and activities of amylases; and (ii) protein concentration and composition, and abundance of N, P, and K. Because our principal interest was to compare information from roots of birdsfoot trefoil, red clover, and sweetclover to that of alfalfa, we conducted this study in a single experiment extending from September through June of the following year. Results obtained for alfalfa in this study are similar to previous findings (Volenec et al., 1991; Boyce and Volenec, 1992b; Hendershot and Volenec, 1993a).

MATERIALS AND METHODS

Plant Culture and Sampling

Three perennial forage legume species, 'Hi-Phy' alfalfa, 'Redland II' red clover, and 'Fergus' birdsfoot trefoil, and 'Norgold' biennial sweetclover were selected for this experiment. Seedlings were established at the Agronomy Research Center, Purdue University, West Lafayette, IN, in early May of 1991. Seeds were sown in 15-m-long rows spaced 92 cm apart in a randomized complete-block with four replications. Resultant plant populations were approximately 40 plants/m of linear row. The silt loam soil (Starks-Fincastle, fine-silty, mixed, mesic, Aeric Ochraqualf) contained 132 kg P ha-1, 230 kg exchangeable K ha-1, and had a pH of 6.0. The soil was limed with pelleted dolomitic lime prior to planting.

Abbreviations: kD, kilodaltons; LSD, least significant difference; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; VSPs, vegetative storage proteins; TNC, total nonstructural carbohydrates; U, unit.
according to soil test recommendations, and was fertilized with an additional 367 kg K ha⁻¹ as KCl. Seeds were inoculated with *Rhizobium meliloti* (alfalfa, sweetclover), *Rhizobium trifolii* (red clover) or *Rhizobium loti* (birdsfoot trefoil) (Liphatech Corp., Milwaukee, WI). Plots were hand-weeded and insects controlled as needed. Plants were defoliated to a 5-cm stubble on 10 July and 10 August. Roots were sampled from 0.5-m sections of row on 10 Sep., 8 Oct., 12 Nov., and 17 Dec. 1991; and 3 and 24 Mar., 27 Apr., and 12 and 21 May 1992. Plants were again defoliated to leave a 5-cm stubble on 2 June 1992, and roots and crowns were immediately sampled with additional samples obtained on 9, 16, 23, and 30 June 1992. Plant samples were taken to a depth of approximately 25 cm and crowns severed from roots. Roots were packed with ice and transported to the laboratory where they were washed free of soil under a stream of cold water. Roots were sliced into 2-cm segments, frozen on solid CO₂, and lyophilized. Root tissues were ground to 1 mm, and were stored at −20°C. Soil temperature data (5-cm depth) from September 1991 through June 1992 are summarized in Table 1. The occurrence of temperatures that killed shoots in autumn (frost) and after shoot growth resumed in spring (freeze) are noted on the figures.

### Sugar and Starch Analysis

Sugars were extracted from 25 mg of root tissue with 1 mL of an 800-mL/L ethanol solution in 1.5-mL microfuge tubes. Tubes were shaken for 10 min at 25°C, microfuged at 14,000 × g for 5 min at 4°C, and the supernatant retained. The ethanol extraction was repeated twice and the combined supernatants diluted to a final volume of 10 mL with ethanol. Sugar concentrations in the ethanol extracts were determined with anthrone (Van Handel, 1968) and glucose as a standard. The ethanol-extracted residue was dried under vacuum at 4°C. Water (500 μL) was added to each tube, and the tubes were heated in a boiling water bath for 10 min to gelatinize starch. The pH of the solution was adjusted to 5.1 by adding 400 μL 0.2 M Na acetate buffer. Starch was digested by adding 0.2 U of amyloglucosidase (Sigma Chemical Co., St. Louis; product A3514 from *Aspergillus niger*) and 40 U of β-amylase (Sigma Chemical Co., St. Louis; product A0273 from *Aspergillus oryzae*) in 100 μL of 0.2 M Na acetate buffer (pH 5.1). Tubes were incubated at 55°C for 24 h with occasional shaking. Preliminary work showed that these conditions were optimal for root starch degradation to glucose with this combination of enzymes (Volenc, 1993, unpublished data). Tubes were centrifuged as before and glucose in the supernatant was determined with glucose oxidase (Glucose Trinder, Sigma Chemical Co., St. Louis; product 315-100). Starch concentration was estimated as 0.9 × glucose concentration.

### Protein Analysis

Procedures in this section were conducted at temperatures between 0 and 4°C unless otherwise stated. Proteins were extracted by suspending 25 mg of root tissue and an equal mass of insoluble polyvinylpolypyrrolidone (Sigma Chemical Co., St. Louis; product P6755) in 750 μL of 100 mM imidazole-HCl buffer (pH 6.5) containing 10 mM 2-mercaptoethanol. Tubes were vortexed, centrifuged at 3600 × g for 10 min, and the supernatant retained. The pelleted was re-extracted once, and the combined supernatants microfuged at 10,000 × g for 10 min. Soluble protein in the supernatant was estimated by means of protein dye-binding (Bradford, 1976). For SDS-PAGE analysis (Laemmli, 1970), protein in 500 μL of supernatant was precipitated overnight with 50 μL of 3.6 M sodium deoxycholate and 50 μL of 4.4 M trichloroacetic acid (Peterson, 1983). Tubes were microfuged at 5000 × g for 5 min, and the pellet resuspended in SDS-PAGE sample buffer. Proteins were separated in 1.5-mm thick gels containing 150 g/L acrylamide and stained with Coomassie Brilliant Blue R-250 (Merril, 1990). Electrophoresis and staining were conducted at room temperature.

### Amylase Assays

Total amylase activity was determined using buffer-extracted proteins diluted to 1 mL with 50 mM imidazole-HCl, pH 7.5. Assays were initiated with the addition of 1 mL of 20 g L⁻¹ amylopectin. Tubes were incubated at 37°C for 5 min and the reaction terminated with the addition of 0.5 mL of dinitrosalicilic acid (Bermfeld, 1955). Color was developed by heating tubes in a boiling water bath for 5 min. Samples were diluted with 10 mL of water and absorbance at 540 nm measured with maltose as a standard. One unit of activity was defined as the quantity of enzyme that released 1 M of maltose min⁻¹. Endoamylase activity was determined by incubating 50 μL of buffer-extracted proteins with 50 L blocked p-nitrophenol maltoheptaoside, an endoamylase specific substrate (McCleary and Sheehan, 1987). Tubes were incubated for 30 min at 40°C and the reaction terminated by adding 2 mL of 1.65 M tris(hydroxymethyl)aminomethane. Absorbance at 410 nm was determined. One unit of activity was defined as the quantity of enzyme that released 1 M of p-nitrophenol min⁻¹.

### Nitrogen, Phosphorus, and Potassium Analysis

Fifty milligrams of root tissue were digested with 5 mL concentrated H₂SO₄, with H₂O₂ added as needed. Total N was determined using micro Kjeldahl analysis. Digested samples were diluted to 75 mL for P and K determination. Mineral P was determined using micro Kjeldahl analysis. Digested samples were diluted with 10 mL of water and absorbance at 760 nm determined. Potassium was assayed by means of atomic emission spectrophotometry.

### Phytate

Phytate was extracted by suspending 1 to 3 g of root tissue in 15 mL of 0.4 M HCl containing 0.7 M Na₂SO₄ for 18 to 24 h at room temperature with stirring. Samples were centrifuged at 10,000 × g for 15 min, and supernatants further clarified by a second centrifugation. Ten milliliters of supernatant were transferred to a 30-mL Corex centrifuge tube and diluted with 10 mL of water, and 5 mL of 15 mM FeCl₃ in
0.2 M HCl containing 0.35 M Na₂SO₄. Tubes were heated in a boiling water bath for 1.5 h. The ferric-phytate precipitate was recovered by centrifuging at 10 000 × g for 10 min and the phytate-P digested and analyzed as described above. Commercial phytate (Sigma Chemical Co., St. Louis; product P-8810) was used as a standard.

**Statistical Analysis**

The experiment was replicated four times and was analyzed as a split-plot design with repeated sampling of plants from within rows over time. Data were analyzed using SAS (SAS Institute, 1989). Variation was partitioned into harvest, species, and replicate main effects and corresponding interactions. Species effects were tested with the species by replicate interaction term. Where F-tests were significant (P < 0.05), an LSD was calculated for mean comparisons.

**RESULTS AND DISCUSSION**

**Total Nonstructural Carbohydrates, Starch, and Sugars**

Root TNC concentrations increased from September to October for birdsfoot trefoil (Fig. 1A). High root TNC were observed for all species in November, with a significant reduction in root TNC concentration occurring by March. Root TNC declined markedly between March and May as shoot growth resumed, then increased, especially in roots of alfalfa and red clover; throughout May until plants were defoliated on 1 June. Defoliation induced a rapid decline in root TNC levels that continued for 2 wk. Root TNC accumulated during the last 2 wk of June, especially in roots of alfalfa and red clover. All species accumulated high levels of starch in roots by early October, with starch concentrations averaging 348, 328, 243, and 238 mg g⁻¹ for alfalfa, sweetclover, red clover and birdsfoot trefoil, respectively (Fig. 1B). Root starch concentrations declined thereafter in all species to less than 50 mg g⁻¹ in March when shoot growth resumed. Starch concentrations increased during May in roots of alfalfa and red clover, but starch levels did not increase significantly in roots of birdsfoot trefoil and sweetclover. After defoliation on 2 June root starch concentrations declined for 2 wk then increased to pre-defoliation levels by Week 4 of shoot regrowth for alfalfa and red clover. Small defoliation-induced changes in starch depletion-reaccumulation occurred in roots of birdsfoot trefoil and sweetclover, observations that agree with previous results (Smith and Graber, 1948; Nelson and Smith, 1968).

As root starch declined in autumn, ethanol-soluble sugar concentrations increased sharply from about 50 mg g⁻¹ in early October to 150 mg g⁻¹ in mid-November (Fig. 1C). Sugar concentrations of sweetclover roots continued to increase gradually throughout winter reaching 288 mg g⁻¹ in March. This sugar concentration was twice that observed in roots of other species. Root sugar concentrations declined in March and April for the perennial legumes, whereas the decline in sugar concentration of sweetclover roots continued until mid-May.

High levels of root TNC are believed necessary for forage legume survival when they are defoliated or exposed to environmental stress (Jung and Smith, 1961; Smith, 1964). Smith (1962) reported similar TNC trends in roots of alfalfa, birdsfoot trefoil, red clover, and sweetclover. A killing frost occurred in late October. Shoot growth resumed in early March and was followed by a killing freeze the second week of March. Herbage was harvested 2 June and roots sampled at weekly intervals thereafter. The least significant difference (LSD, P < 0.05) is provided for comparison of species × harvest interaction means.

![Seasonal changes in concentrations of total nonstructural carbohydrate (TNC, A), starch (B), and sugar (C) in roots of alfalfa, birdsfoot trefoil, red clover, and sweetclover. A killing frost occurred in late October. Shoot growth resumed in early March and was followed by a killing freeze the second week of March. Herbage was harvested 2 June and roots sampled at weekly intervals thereafter. The least significant difference (LSD, P < 0.05) is provided for comparison of species × harvest interaction means.](image-url)
of sugar accumulation in roots of forage legumes, and the physiological mechanism(s) facilitating this process.

**Amylase Activities**

To better understand the biochemical basis for starch degradation in roots of these species we examined specific activities of root amylases. Total amylase specific activity is comprised primarily (>99%) of exoamylase activity (β-amylase; Doehlert et al., 1982) Exoamylase specific activity is of special interest because alfalfa roots contain large quantities of this enzyme (Boyce and Volenec, 1992a). However, total amylase specific activities in our previous work declined in alfalfa roots after defoliation and in spring; periods of rapid root starch degradation (Volenec and Brown, 1988; Volenec et al., 1991). Total amylase specific activity differed markedly among species. Total amylase specific activities, averaged over harvests, were 56.4, 22.5, 2.9, and 2.7 U/mg protein for alfalfa, red clover, birdsfoot trefoil and sweetclover, respectively (Fig. 2A). The specific activity of total amylase from alfalfa is similar to that reported in a previous study where we purified root exoamylase (Boyce and Volenec, 1992a). The low total amylase activity observed in birdsfoot trefoil roots agrees with previous findings from experiments with greenhouse-grown plants (Boyce et al., 1992). Total amylase specific activity did not change during autumn and winter even though root starch degradation was occurring (Fig. 1B). Total amylase specific activity declined in roots of alfalfa and red clover between 27 April and 12 May when shoot growth was rapid. The large differences in total amylase specific activity among species that exhibited a similar pattern of root starch utilization suggests that the high exoamylase activity that constitutes most of the high total amylase specific activity present in alfalfa and red clover roots may not be required for starch hydrolysis. Results of purification and characterization studies (Boyce and Volenec, 1992a) indicate that exoamylase is very abundant in alfalfa roots, comprising 8% of root soluble proteins. The preponderance of this enzyme in roots of alfalfa and red clover, along with the preferential decline in specific activity when rapid shoot growth resumes in spring (Fig. 2A), is indicative of a VSP. Confirmation that exoamylase may function as a VSP in roots of these species awaits further research.

Endoamylase activity has been previously correlated with starch lost from roots of alfalfa and birdsfoot trefoil (Volenec et al., 1991; Boyce et al., 1992). Endoamylase activity of alfalfa roots was lower than that of red clover and sweetclover in autumn and winter (Fig. 2B). Endoamylase activity remained unchanged throughout winter in all species even though root starch was being degraded. This inconsistency has been observed previously for alfalfa roots during winter (Volenec et al., 1991). The poor association of endoamylase activity and root starch degradation in winter could be explained if starch degradation is rapidly occurring only in a small number of root cells at any one time as we have previously shown (Habben and Volenec, 1990). Under these circum-

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**Fig. 2.** Seasonal changes in specific activity of total amylase (primarily exoamylase, A) and endoamylase (B) in root extracts from alfalfa, birdsfoot trefoil, red clover, and sweetclover. A killing frost occurred in late October. Shoot growth resumed in early March and was followed by a killing freeze the second week of March. Herbage was harvested 2 June and roots sampled at weekly intervals thereafter. The least significant difference (LSD, P < 0.05) is provided for comparison of species × harvest interaction means.

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**Fig. 3.** Seasonal changes in concentrations of nitrogen (N, A) and buffer-soluble protein (B) in roots of alfalfa, birdsfoot trefoil, red clover, and sweetclover. A killing frost occurred in late October. Shoot growth resumed in early March and was followed by a killing freeze the second week of March. Herbage was harvested 2 June and roots sampled at weekly intervals thereafter. The least significant difference (LSD, P < 0.05) is provided for comparison of species × harvest interaction means.
stances, the high endoamylase activity of a few cells is masked by the relatively low activity present in most other cells. As shoot growth resumed in March, endoamylase activity increased substantially. Endoamylase activity decreased in alfalfa and red clover roots in May when starch reaccumulated in roots. However, endoamylase activity remained high and starch concentrations low in birdsfoot trefoil and sweetclover roots during the remainder of the study. Endoamylase activity in alfalfa and red clover roots increased during starch degradation that occurred after defoliation on 2 June. This result agrees with previous findings with alfalfa (Volenec et al., 1991; Boyce et al., 1992) and is consistent with the view that endoamylase is an important enzyme controlling starch hydrolysis (Dunn, 1974).

**Root Nitrogen and Protein**

Root N concentrations of sweetclover were higher in September and October than those observed for the other species (Fig. 3A). Root N concentrations increased in roots of all species from September through mid-December, and then decreased from mid-March through May in roots of alfalfa, red clover and birdsfoot trefoil. A large increase in N concentration was observed for sweetclover roots in April, followed by a rapid decline. Shoot removal on 2 June resulted in additional declines in root N of birdsfoot trefoil and sweetclover; however, these changes were smaller in magnitude when compared with declines observed following initiation of shoot growth in spring.

Protein concentrations were highest in alfalfa roots in autumn (Fig. 3B). Root protein concentrations of all species increased until October or early November, then remained relatively constant over winter. As shoot growth resumed in spring, root protein concentrations of all species declined. Proteins in birdsfoot trefoil roots declined to low concentrations in late-May, and remained low (12 mg g\(^{-1}\)) throughout the growing season. Proteins reaccumulated in roots of alfalfa, and red clover in late May, then declined for 2 wk in roots of all species except birdsfoot trefoil after defoliation on 2 June. This defoliation-induced cyclic pattern of root protein loss followed by reaccumulation during late shoot regrowth is similar to that observed in previous alfalfa research (Hendershot and Volenec, 1993b). Birdsfoot trefoil root protein concentrations did not change after defoliation.

SDS-PAGE was used to examine qualitative changes in root protein composition (Fig. 4). Polypeptide composition of species differed. Alfalfa roots accumulated three polypeptides with molecular weights of 32, 19, and 15 kD, and β-amylase (57 kD) in autumn. These polypeptides were present in roots in early March, but declined after shoot growth resumed (Fig. 4, alfalfa-Lanes 3 and 4). Previous work in our laboratory suggests that these polypeptides in alfalfa roots function as VSPs (Volenec et al., 1991; Hendershot and Volenec, 1993a,b). Studies using \(^{15}\)N labeling of root N pools indicate that these
proteins are preferentially degraded and the N redistributed to regrowing shoots (Barber et al., 1996). Other changes in polypeptide composition also were observed including June samples of red clover roots where a 24-kD polypeptide accumulated (Fig. 4, Lanes 5–7). However, polypeptides fulfilling the criteria for being VSPs (Cyr and Bewley, 1990) were not observed in root protein extracts of red clover, sweetclover, or birdsfoot trefoil, indicating that VSP accumulation is not required for winter survival and spring growth. All species accumulated a 17-kD polypeptide in roots by December (Fig. 4, Lane 2) that remained until March (Fig. 4, Lane 3), but subsequently disappeared. The nature of this polypeptide remains unknown, but its uniform appearance in roots of all species in autumn and disappearance in spring suggests it may have a role in cold acclimation.

**Phosphorus and Phytate**

Root P concentrations followed similar trends in autumn for all species (Fig. 5A). Root P concentrations declined slightly in October, and then increased gradually until late April. Root P concentrations declined dramatically in May. Unlike starch and protein, root P concentrations were not altered in a consistent pattern by defoliation. Averaged over harvests, alfalfa generally had the lowest root P levels throughout the study compared with other species, while birdsfoot trefoil maintained relatively high root P levels, particularly during late spring and early summer (Fig. 5).

Phytate (myo-inositol hexakisphosphate), a storage form of P common to seeds, was present in roots of all species (Fig. 5B). Concentrations of phytate-P differed markedly among species, with higher concentrations observed in roots of trefoil at most harvests. Phytate-P increased between October and December in roots of all species except alfalfa. Phytate-P increased between December and March in roots of alfalfa, red clover and birdsfoot trefoil. By March, phytate-P levels in birdsfoot trefoil roots represented as much as 12% of root total P, but only accounted for 5 to 8% of total P in roots of other species (Fig. 5C). Our values are lower than those of Campbell et al. (1991) who reported that phytate-P represented 10 to 15% of total P in alfalfa root and crown tissues. Our lower values may be due to our use of roots and exclusion of crowns from the analysis. Phytate-P declined when shoot growth resumed in spring. The proportion of P in phytate declined earlier than total P suggesting that phytate-P may serve as a storage form of P in roots of these species.

**Potassium**

With the exception of red clover, root K concentrations declined between September and November (Fig. 6). Between December and mid-May root K concentrations increased; a pattern distinct from the large declines noted for root N or P concentrations. In alfalfa and red clover roots, K concentrations declined slightly between mid-May and early June, a time of rapid starch accumulation in roots of these species. Analysis of root K concentrations on a structural dry weight basis (dry weight minus root TNC) revealed that dilution of root K by the accumu-
lating starch contributed to much of this decline (data not shown). Alfalfa exhibited lower root K concentrations compared to other species at nearly all samplings, while sweetclover roots contained high K concentrations from mid-May through June. Application of K is thought to increase plant winterhardiness and tolerance to adverse environmental conditions (Calder and MacLeod, 1966; Adams and Twersky, 1960). Evidence from this study shows that, unlike other root constituents studied, root K does not accumulate to high concentrations during winter hardening. In addition, K concentration increased markedly in spring when shoot growth resumed; a period when concentrations of other root constituents declined.

**CONCLUSIONS**

Proteins, N, P, and phytate in roots of these forage legume species accumulated during winter hardening, and were depleted from roots when shoot growth resumes in spring. Root protein composition differed among species, especially with regard to the presence of putative VSPs in alfalfa, and high total (exoamylase) amylase specific activity in both alfalfa and red clover. Understanding key features of the winter hardening process may facilitate genetic improvement for winter survival of forage legumes.

**REFERENCES**


