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Seasonal changes in tannin and nitrogen contents of *Casuarina equisetifolia* branchlets*

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Abstract: Seasonal dynamics of total phenolics (TP), extractable condensed tannins (ECT), protein-bound condensed tannins (PBCT), fiber-bound condensed tannins (FBCT), total condensed tannins (TCT), and protein precipitation capacity (PPC) in young, mature and senescent branchlets of *Casuarina equisetifolia* were studied at Chishan Forestry Center of Dongshan County, Fujian Province, China. In addition, nitrogen contents of branchlets at the different developmental stages were also determined. The contents of TP and ECT, and PPC in young branchlets were significantly higher than those in mature and senescent branchlets through the season. However, PBCT contents were significantly higher in senescent branchlets than those in young and mature branchlets; FBCT fluctuated with season. Young branchlets had the highest N content, which decreased during branch maturity and senescence. The highest contents of TP and the lowest contents of TCT and N in young and mature branchlets were observed in summer. There was a significant negative correlation between TP and N contents. In contrast, TCT contents were positively correlated to N contents. Nutrient resorption during senescence and high TCT:N ratios in senescent branchlets are the important nutrient conservation strategies for *C. equisetifolia*.

Key words: *Casuarina equisetifolia*, Condensed tannins, Total phenolics, Nitrogen, Seasonal dynamics
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INTRODUCTION

Casuarina equisetifolia is a nitrogen-fixing tree of considerably social, economic and environmental importance in tropical/subtropical littoral zones of Asia, the Pacific, and Africa. It is commonly used in agro-forestry systems for soil stabilization and reclamation work and in coastal protection and rehabilitation (Pinyopusarerk and House, 1993; Pinyopusarerk and Williams, 2000). It is one of the most extensively introduced tree species outside its natural range. There are more than 300000 ha of *C. equisetifolia* plantations in the coastline of southern China (Zhong *et al*., 2005). Despite the widespread planting and known ecological and physiological properties of *C. equisetifolia*, very little has been done to explore secondary metabolism production along with seasonal changes, although they are recognized as high in tannin and astringent (Okuda *et al*., 1980).

Tannins play a role in a number of ecological processes in addition to herbivore defense, including litter decomposition, nutrient cycling, nitrogen sequestration, microbial activity, humic acid formation, metal complexation, and pedogenesis (Kraus *et al*., 2003). Tannins comprise a significant portion of terrestrial biomass C (Hernes *et al*., 2001). Leaves and bark may contain up to 40% tannin by dry weight

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(Kuiters, 1990; Matthews *et al*., 1997; Lin *et al*., 2006; 2007), and in leaves and needles tannin contents can exceed lignin levels (Hernes *et al*., 2001). Because tannins are complex and energetically costly molecules to synthesize, their widespread occurrence and abundance suggest that tannins play an important role in plant function and evolution (Cates and Rhoades, 1977; Zucker, 1983). Some species rich in polyphenols appear to gain a competitive advantage by altering nutrient cycling and thus influencing forest dynamics (Northup *et al*., 1998; Fierer *et al*., 2001; Kraus *et al*., 2003). Plants greatly influence soil processes through litter inputs (Aber *et al*., 1990); thus, alterations in foliar chemistry have important implications for plant-litter-soil interactions and ecosystem function (Kraus *et al*., 2004).

Traditionally, justification for the high metabolic cost associated with the production of tannins was attributed to improve herbivore defense (Feeny, 1970). Two major hypotheses have been proposed to predict the effects of environmental factors on secondary metabolite concentrations. The carbon-nutrient balance (CNB) hypothesis postulates that phenolic levels in plants are determined by the balance between carbon and nutrient availability (Bryant *et al*., 1983). The growth-differentiation balance (GDB) hypothesis (Loomis, 1932; Lorio, 1986; Herms and Mattson, 1992) considers factors that limit growth and differentiation (the sum of chemical and morphological changes that occur in maturing cells, including carbon-based secondary synthesis). The production of phenolics dominates when factors other than photosynthate supply are suboptimal for growth (e.g., under nutrient limitation or moderate drought). Resource-based theories assume that the synthesis of defensive compounds is constrained by the external availability of resources and internal trade-offs in resource allocation between growth and defense (Riipi *et al*., 2002). They state that growth processes dominate over the production of defensive compounds, and that more carbon is left for defensive compounds only when plant growth is restricted by a lack of mineral nutrient (emphasized by the CNB hypothesis) or by any factor (according to the GDB hypothesis) (Haukioja *et al*., 1998). Jones and Hartley (1999) presented a protein competition model (PCM) for predicting total phenolics allocation and content in leaves of terrestrial higher plants. They stated that "protein and phenolics synthesis compete for the common, limiting resource phenylalanine," so nitrogen (N) rather than C is the limiting resource for synthesis of phenolics.

Despite the growing knowledge of the physiological basis and ecological consequences of leaf phenolics presence in plant tissues, only a few extensive datasets are available to evaluate the relative importance of leaf phenolics variability caused by environmental changes and developmental stages (Covelo and Gallardo, 2001; Lin *et al*., 2007). This variability may determine not only the susceptibility of plants to herbivore attack, but also important aspects of nutrient cycling in terrestrial and aquatic ecosystems (Gallardo and Merino, 1992; Serrano, 1992; Mafongoya *et al*., 1997; Northup *et al*., 1998).

According to PCM, the growth-defense trade-off depends not only on competition for a limited pool of available carbohydrates, but also on competition for nitrogen as a component of common precursor compounds (Jones and Hartley, 1999; Gayler *et al*., 2007). In this regard, information on the seasonal dynamics of tannins and nitrogen contents is necessary.

The objective of the present study was to test the validity of the following hypotheses: (1) Tannins of branchlets at different developmental stages follow a seasonal pattern; (2) The production of phenolics dominates under nutrient limitation. To answer these hypotheses, a field investigation of *C. equisetifolia* was conducted at Chishan Forestry Center of Dongshan County, Fujian Province, China.

MATERIALS AND METHODS

Study areas

The study was carried out at Chishan Forestry Center of Dongshan County (23°40′ N, 117°18′ E), Fujian Province, China. The climate of the region belongs to southern subtropical maritime monsoon climate, with annual temperature ranging from 3.8 °C to 36.6 °C. Mean annual precipitation and evaporation are 1103.8 mm and 2027.9 mm, respectively. The rain season is from May to September and the dry season is from October to April of the next year. The soils are coastal sandy barren.

The *C. equisetifolia* plantations were artificial, pure forests that were planted in 1999. The coverage of dense forest was 0.7, tree density was 3552 tree/ha, canopy height ranged from 5~7 m, and diameter at breast height (DBH) was (8.1±1.4) cm. The average N and P contents of the soils at 20 cm depth were (54.49±7.89) and (2.69±0.42) mg/kg, respectively, and soil pH was 4.19±0.05.

Materials

Thirty individuals of *C. equisetifolia* were chosen and labeled. The height and growth conditions of the chosen trees were similar. The development stages of branchlets were demarcated into three stages, i.e., young branchlets (newly-emerged, usually shorter than 5 cm in length at the top of branch, and light green in color), mature branchlets (fully developed, usually 15~25 cm long, and dark green in color), and senescent branchlets (old branchlets, white or grey in color). Branchlets damaged by insects and disease or mechanical factors were avoided. Young, mature and senescent branchlets of each labeled tree were collected in March, June, September, and December of 2007. All samples were taken to the laboratory immediately after sampling and cleaned with distilled water.

Chemical analyses

All chemicals were of analytical reagent purity grade. An additional standard denoted here as purified tannin, was extracted from *C. equisetifolia* branchlets and purified on Sephadex LH-20 (Amersham, USA) according to the procedure previously described by Asquith and Butler (1986) as modified by Hagerman (2002). The condensed tannin standard was freeze-dried and stored at −20 °C until required.

Procedures described by Lin *et al.*(2006) were used to determine total phenolics (TP), extractable condensed tannins (ECT), protein-bound condensed tannins (PBCT), fibre-bound condensed tannins (FBCT), and protein precipitation capacity (PPC). TP were measured with the Prussian blue method (Graham, 1992), and ECT, PBCT and FBCT were assayed by the butanol-HCl method (Terrill *et al.*, 1992) using purified tannins from *C. equisetifolia* branchlets as the standard. The contents of total condensed tannins (TCT) were calculated by adding the respective quantities of ECT, PBCT, and FBCT (Terrill *et al.*, 1992). A radial diffusion assay was used to determine the PPC (Hagerman, 1987).

N content was determined with Nessler's reagent after Kjeldahl digestion of powdered samples with sulfuric acid and hydrogen peroxide (Mae *et al.*, 1983).

Calculations

Resorption efficiency (RE) was calculated as the percentage of N recovered from the senescing leaves (Aerts, 1996; Killingbeck, 1996):

$$RE\ (\%)=(A_1-A_2)/A_1\times100,$$

where A_1 is N content in mature branchlets; A_2 is N content in senescent branchlets.

Statistical analyses

All measurements were replicated three times and analyzed using one-way analysis of variance (ANOVA) (SPSS 11.0 for Windows) with TP, ECT, PBCT, FBCT, TCT, and PPC.

RESULTS

Seasonal dynamics of total phenolics contents

TP content was highest in young branchlets, (146.17±4.14) to (169.23±4.78) mg/g, and decreased with maturity and senescence. TP did not follow the identical pattern for young, mature, and senescent branchlets. TP contents of young [(169.23±4.78) mg/g] and mature [(118.40±5.81) mg/g] branchlets in summer were relatively high than those in other seasons. TP content of senescent branchlets was the lowest in summer [(24.56±0.92) mg/g] and the highest in winter [(98.74±16.10) mg/g] (Fig.1).

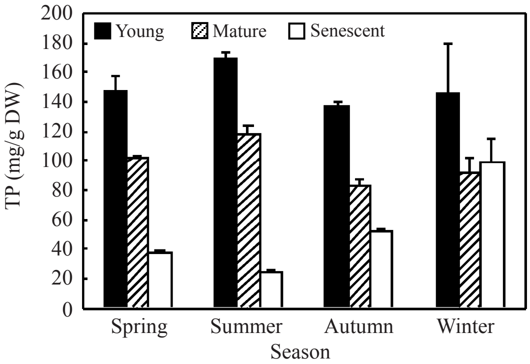

Fig.1 Seasonal changes in total phenolics contents of branchlets at different development stages of *C. equisetifolia*

Seasonal dynamics of condensed tannin contents

ECT contents of young and senescent branchlets were significantly higher in winter than in other three seasons (Fig.2a), which was different from the observation of ECT contents of mature branchlets. The ECT contents of young and senescent branchlets in winter reached (316.17±64.10) and (168.06±44.19) mg/g, respectively. ECT contents of mature branchlets ranged from (101.44±16.17) to (137.36±13.48) mg/g, and remained unchanged with season. ECT contents of senescent branchlets were significantly lower than those of young and mature branchlets except for the mature branchlets in winter, showing that the ECT contents of the branchlets decreased with senescence.

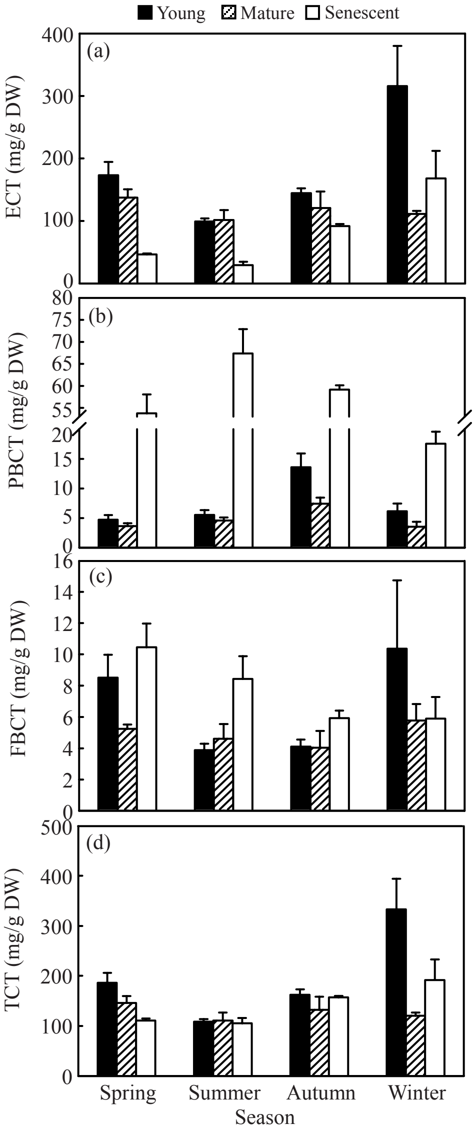

Fig.2 Seasonal changes in ECT (a), PBCT (b), FBCT (c) and TCT (d) contents of branchlets at different development stages of *C. equisetifolia*

PBCT contents were significantly higher in senescent branchlets than in young and mature branchlets (Fig.2b), showing that PBCT contents increased with senescence. The highest PBCT contents of young and mature branchlets in autumn were (13.55±2.32) and (7.43±1.01) mg/g, respectively. PBCT contents of young and mature branchlets both increased gradually during the growing season and then declined in winter. However, FBCT contents of the branchlets showed the decreasing trends during the growing seasons, and then increased in winter (Fig.2c). Except for in winter, FBCT contents of senescent branchlets were significantly higher than those of young and mature branchlets (Fig.2c).

PBCT was significantly higher than FBCT for senescent branchlets, but there was no significant difference for young and mature branchlets. ECT accounted for 89%~95% of TCT in young and mature branchlets, with 5%~11% being bound to protein or fibre (Fig.2d).

Seasonal dynamics of protein precipitation capacity

PPC was the highest in young branchlets, and decreased with maturity and senescence (Fig.3). The PPCs of young [(530.19±30.39) cm^2/g] and mature [(398.28±23.01) cm^2/g] branchlets were lower in spring than in other seasons.

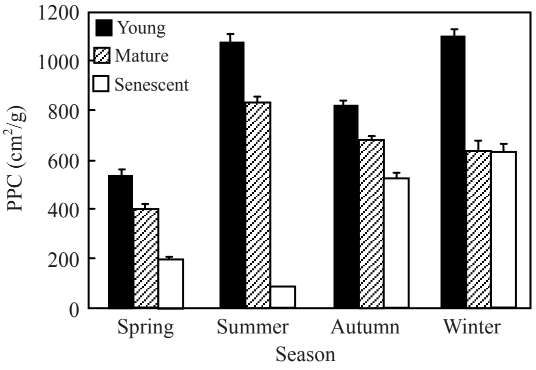

Fig.3 Seasonal changes in PPC of branchlets at different development stages of *C. equisetifolia*

Seasonal dynamics of nitrogen content and resorption efficiency

Young and mature branchlets had significantly higher nitrogen contents than senescent branchlets. The N contents of senescent branchlets were all below 0.86% (Fig.4). The lowest N contents all occurred in the summer for young, mature, and senescent branchlets. The N contents remained relatively stable for the other seasons. Nitrogen resorption efficiency (NRE) ranged from (51.01±3.94)% to (63.00±8.61)% with no significant difference among four seasons.

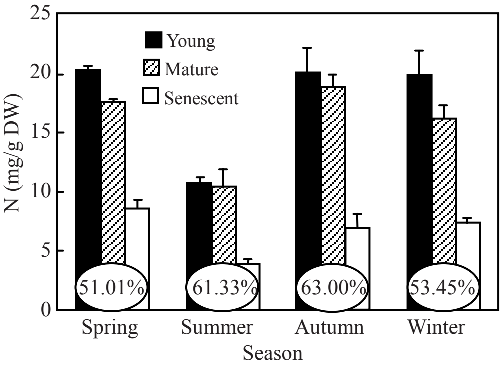

Fig.4 Seasonal changes in N contents of branchlets at different development stages of *C. equisetifolia*
Numbers within the ellipses represent the nitrogen resorption efficiency

DISCUSSION

Young branchlets had the higher contents of TP and ECT than mature and senescent branchlets. The observed changes in TP and ECT contents of the branchlets associated with different development stages are in accordance with the findings reported for different mangrove species (Lin *et al*., 2006; 2007) and oak species (Makkar *et al*., 1988; Rossiter *et al*., 1988). Young leaves need to be well defended, since damage to young leaves may cause a larger decline in future photosynthesis than damage to mature leaves (McKey, 1974; Harper, 1989). In addition, the fact that young branchlets contain much higher contents of TP and ECT compared with mature and senescent branchlets may indicate that young branchlets are experiencing more intense selective pressure than mature and senescent branchlets. Many of the soluble carbon compounds (which also include polyphenols) are expected to be translocated from leaves during senescence (Mafongoya *et al*., 1998). The depletion of TP and ECT during senescence in the spring, summer and autumn and the enrichment of TP and ECT during senescence in winter were found in the present study. The decrease in phenolics content may reflect an active turnover of phenolics and an increase in bound or nonextractable phenolics during senescence (Fig.2b). As polyphenols are water-soluble and susceptible to leaching (Hättenschwiler and Vitousek, 2000), leaching of polyphenols or tannins by sporadic rain (from green leaves) might be a cause for the net ‘enrichment’ in senesced leaves (Teklay, 2004), but the exact cause or mechanism is difficult to ascertain. Constantinides and Fownes (1994) also found both an increase and a decrease in polyphenol contents. Kuhajek *et al*.(2006) observed that no significant effects of leaf age on both condensed tannins and total phenolics. As for bound condensed tannins, PBCT contents were significantly higher in senescent branchlets than in young and mature branchlets.

Seasonal changes in leaf chemistry reflect changing demands for carbohydrates and nutrients resulting from normal growth and differentiation processes (Wareing, 1959; Moorby and Wareing, 1963). The GDB hypothesis (Loomis, 1932; Lorio, 1986; Herms and Mattson, 1992) and the CNB hypothesis (Bryant *et al*., 1983) assume that the synthesis of carbon-rich secondary chemicals is limited by the availability of photosynthesis (carbon). According to these hypotheses, growth processes dominate over differentiation or production of carbon-rich secondary compounds as long as conditions are favorable for growth, but the GDB hypothesis is associated with the temporal variation in growth activity more directly than the CNB hypothesis (Tuomi, 1992). If plant growth is active, and therefore demands large amounts of carbon, allocation to carbon-rich secondary metabolites, e.g., phenolics, is predicted to decline; however, when growth is limited more than photosynthesis, allocation to defense will increase (Riipi *et al*., 2002). Under the assumptions of the GDB hypothesis, allocation to phenolics should be low in spring during rapid growth of the short-shoot leaves (Riipi *et al*., 2002). The amounts of phenolics should increase most quickly in summer, when the photosynthetic capacity of the newly matured leaves is highest (Mooney and Gulmon, 1982). The highest TP content in summer was found in this paper (Fig.1). *C. equisetifolia* growth is active from May to September (rain season) with the relatively high soil water availability. According to the PCM hypothesis, protein demand should be highest when plant grows rapidly, and allocation to those phenolics that are derived from phenylalanine should simultaneously decrease (Jones and Hartley, 1999), as phenylalanine is the common precursor of either protein or condensed tannins synthesis (Hättenschwiler and Vitousek, 2000). However, the second major group of phenolics, the hydrolysable tannins, has gallic acid as its precursor (Haukioja *et al.*, 1998). Therefore, depending on the relative strength of the synthetic route via dehydroshikimic acid to gallic acid, hydrolysable tannins may or may not trade off directly with protein synthesis (Haukioja *et al.*, 1998). Hydrolysable tannins are thought to be metabolically cheaper than phenylpropanoids, and the synthesis of it in the season may be a cost-saving defense strategy during a time when condensed tannins are not yet effective as defense (Haukioja *et al.*, 1998; Salminen *et al.*, 2001). However, the changes in contents do not necessarily reflect the quantitative allocation of tannins to the leaves, because of rapid turnover of labile compounds (Kleiner *et al.*, 1999) and because the contents are affected by concomitant changes in proportions of other components of the leaves, e.g., structural leaf components (Koricheva, 1999).

The lowest nitrogen contents of branchlets at the different development stages occurred in summer, when *C. equisetifolia* growth is active. Similarly, Aerts *et al.*(1999) suggested that summer warming reduced N contents of mature and senescent leaves in *Rubus*. First, a portion of N was allocated to other portions (e.g., roots and flowers); for example, the peak of the flowering period appears from April to June for *C. equisetifolia* (Morton, 1980). Second, N contents were diluted by branchlets mass accumulation during summer when *C. equisetifolia* grew rapidly. Changes in leaf N contents may directly impact the photosynthetic capacity of the species involved, as there is usually a direct relation between leaf N content and the maximum rate of photosynthesis (Lambers *et al.*, 1998). In this study, TP contents were inversely related to N contents. It is common to find a negative correlation between N and secondary compound contents, such as phenolics and tannins (Horner *et al.*, 1987; Mansfield *et al.*, 1999). This pattern lends to support source-sink hypotheses, such as the CNB hypothesis (Bryant *et al.*, 1983) and the GDB hypothesis (Herms and Mattson, 1992) that predict increased C allocation to secondary C compounds under low nutrient conditions. However, there was a positive correlation between N and TCT contents, which is consistent with a previous study (Horner *et al.*, 1987) but inconsistent with other study (Kraus *et al.*, 2004). They may result from differences in the carbon:nutrient balance of the plants resulting from differences in relative resource availability (Horner *et al.*, 1987).

The young leaves were likely to produce toxic effects even at low levels of intake, and PPC was considered to be related to the biological activity (Martin and Martin, 1982; Deshpande *et al.*, 1986) and was very high in the young leaves. The research on *C. equisetifolia* comes to the same result (Fig.3). The capacity of tannin to bind proteins was related to the molecular size of the tannins (Makkar *et al.*, 1987). In general, it was found that the larger-sized condensed tannin could precipitate more protein than the smaller-sized condensed tannin (Osborne and McNeill, 2001). In this study, there was no significant correlation between TP or TCT and PPC in young and mature branchlets (Table 1), which is consistent with the previous study (Martin and Martin, 1982). However, there was a significantly positive correlation between TP or TCT and PPC in senescent branchlets. The results indicate that the possible increase in degree of polymerization with senescence leads to the more larger-sized tannins and the more protein precipitated.

TP:N ratio was the highest in young branchlets and decreased with maturity and senescence in spring and summer, while TP:N ratio of branchlets decreased with maturity and increased with senescence in autumn and winter (Fig.5a). TP:N ratios of young and mature branchlets were both the highest in

Table 1 Correlative coefficient of selected branchlet data (n=12)

Branchlets	TP vs N	TCT vs N	TP vs TCT	TP vs PPC	TCT vs PPC
Young	−0.615 (P=0.033)	0.554 (P=0.061)	−0.081 (P=0.802)	0.190 (P=0.554)	0.242 (P=0.448)
Mature	−0.785 (P=0.002)	0.497 (P=0.100)	−0.126 (P=0.695)	0.147 (P=0.649)	−0.647 (P=0.023)
Senescent	0.374 (P=0.231)	0.264 (P=0.407)	0.926 (P=0)	0.870 (P=0)	0.870 (P=0)

summer, and remained relatively stable in other three seasons. The relatively low TP contents of senescent branchlets paired with high N contents in spring resulted in the low TP:N ratios. The TP:N ratios of senescent branchlets increased through the season (from spring to winter).

Seasonal changes in TCT:N ratios during development stages were different from the observation of TP:N ratios (Fig.5b). TCT:N ratios of senescent branchlets were significantly higher than those of young and mature branchlets.

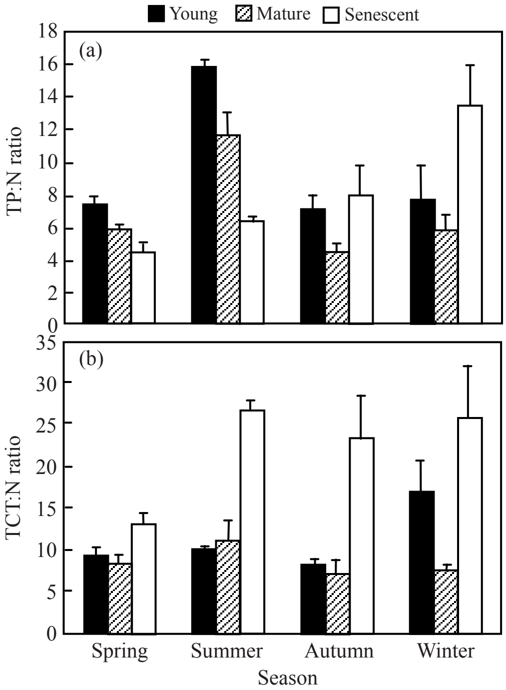

Fig.5 Seasonal changes in TP:N (a) and TCT:N (b) ratios of branchlets at different development stages of *C. equisetifolia*

TCT:N ratios of senescent branchlets were significantly higher than TP:N ratios of senescent branchlets in corresponding seasons.

The role that tannins play in soil processes is believed to occur largely through their ability to precipitate proteins, as well as their relative resistance to decomposition (Kuiters, 1990; Kraus *et al*., 2003). The amount of tannins entering the soil relative to the amount of proteins or N may be the key factor influencing soil nutrient cycling. Therefore, parameters such as TP:N and CT:N ratios may be the best predictors of litter quality (Kraus *et al*., 2004). In green foliage, high TP:N and TCT:N ratios may help reduce herbivory. In our study, TP:N ratios in young branchlets were higher than those in mature branchlets. The highest levels of TP:N in young and mature branchlets both occurred in summer, indicating potentially increased grazer deterrence. TP:N ratios in senescent branchlets increased through the season (from spring to winter). TCT:N ratios were significantly higher in senescent branchlets than those in young and mature branchlets. Nutrient resorption (about 50%~60%) during senescence and high TCT:N ratios in senescent branchlets are the important nutrient conservation strategies for *C. equisetifolia*.

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