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Soil Enzyme Activities in *Pinus tabuliformis* (Carriére) Plantations in Northern China

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Abstract: Changes in forest stand structure may alter the activity of invertase, urease, catalase and phenol oxidase after thinning *Pinus tabuliformis* (Carriére) plantations in Yanqing County of Beijing, China. We examined changes in these soil enzymes as influenced by time since thinning (24, 32, and 40 years since thinning) for 3 seasons (spring, summer and autumn) following harvesting at two depths in the mineral soil (0–10 cm and 10–20 cm). Invertase and urease increased significantly with time since thinning. Catalase activity was highest in the 24-year-old stand and there were no statistically significant differences between the 32- and 40-year-old stands. In addition, maximum invertase, urease, catalase, and phenol oxidase activities occurred during the summer; minimum activities occurred in autumn. Invertase and urease were positively correlated with each other, as were catalase and phenol oxidase. Most soil enzyme activity was higher in the 0–10 cm layer than at the 10–20 cm depth. As time from thinning increased, differences among soil depth became less significant. These results suggest that seasonal changes of these enzymes have different roles, as the time since thinning and thinning treatments may have both short- and long-term impacts on soil microbial activity.

Keywords: invertase; urease; catalase; phenol oxidase; forest thinning

1. Introduction

Soil enzymes are an essential component of forest ecosystems because they play a role in catalyzing the reactions necessary for organic matter decomposition and nutrient cycling [1,2]. Previous studies have demonstrated that soil biochemical parameters, such as soil enzymes, can be sensitive indicators of ecosystem stress, changes in forest health, or sustainability and overall soil quality [3–5]. In addition, forest management activities may alter enzyme activities [6]. In forest ecosystems, bacteria and fungi are responsible for extracellular synthesis and secretion of enzymes, such as proteases, ureases, and pectinases, and constitute an important part of the soil matrix [7]. Extracellular enzymes also contribute to regulating the decomposition of both the forest floor and mineral soil organic matter and, therefore, soil carbon storage [8,9]. The activity of soil enzymes can be sensitive indicators of ecosystem stress and can also serve as measures of forest health and the sustainability of managed ecosystems [4,6]. In addition, since soil biological and biochemical properties can respond rapidly to forest harvesting, enzyme activity can be used to indicate changes in soil quality [10]. After being synthesized, enzymes are stabilized through association with humic substances [11]. Generally,

extracellular enzymes responsible for the degradation of different soil organic matter fractions are separated into two groups; hydrolytic enzymes (responsible for the decomposition of labile organic matter to acquire nutrients for primary metabolism) and oxidative enzymes (responsible for the degradation of recalcitrant organic matter for co-metabolic acquisition of nutrients) [12,13] Despite numerous studies [14–16], the results obtained by different researchers are often contradictory [17–19], and soil microbes are sensitive to changes in both aboveground and belowground environmental changes [20]. Forest plantations account for a large portion of the global terrestrial ecosystem carbon sink [21,22], and thinning of these forests alters both soil and stand characteristics, giving rise to the inconsistent patterns of extracellular enzyme activities in litter or mineral soil as stands age [23–26]. Although many studies have shown that soil enzymatic activity can be related to stand management, soil quality, or ecosystem stress [6,10,27], the results may be site specific. For example, in a boreal forest chronosequence after a wildfire in Alaska, two recent studies reported that oxidative enzyme activities decreased with stand age, while hydrolytic enzyme activities did not change significantly [24] or hydrolytic enzymes increased with stand age [25]. Little is known about the impact of thinning on changes in mineral soil enzyme activity.

In China, there are 0.69 million hectares of forest plantations, many of them consisting of Chinese pine (*Pinus tabulaeformis* Carrére). Chinese pine is widely distributed in China and has an important ecological role. Water quality, temperature, plant photosynthetic capacity and growth, and litterfall within Chinese pine plantations all vary depending on the season and time since thinning [28]. It is important, therefore, to understand the role of extracellular enzymes in nutrient release and organic matter decomposition to maintain productive Chinese pine stands and to maintain soil quality [10]. In our study, four kinds of soil enzymes (invertase, urease, phenol oxidase and catalase) were selected for analysis because of their roles in carbon (C) and nitrogen (N) cycling and organic matter (OM) decomposition in forest soils. Microorganisms use phenol oxidases to degrade lignin and humus to gain C and other nutrients, but this has only been reported in a small subset of soil enzyme studies [29]. Invertase is also important for decomposition processes and may be related to stand succession after harvest [30]. Urease activity is associated with root and rhizosphere changes after harvesting. Therefore, urease activity is associated with changes in OM inputs and alteration of soil pH [31]. Catalase activity is usually higher in high quality soils and activity may decrease or cease when soil pH, nutrients, or temperature extremes occur [32].

We hypothesized that these soil enzymes (phenol oxidase, invertase, catalase, and urease) will exhibit seasonality based on their function in the soil, and that the combination of overstory removal and season (e.g., soil temperature) will produce distinct changes in their activity at two soil depths. Consequently, our objectives were to determine how time since thinning might alter invertase, urease, catalase and phenol oxidase enzyme activities in a northern Chinese plantation.

2. Materials and Methods

2.1. Study Area

The study was performed in the northern part of Yingpan village in Liubinbu township, Yanqing county, Beijing (116°16′ E, 40°35′ N), China. This site is in the warm temperate zone with a semi-humid continental monsoon climate. The mean annual temperature is 8.8 °C (min. −9.8 °C and max. 20.9 °C) and the average frost-free period is 144 days. Rainfall averages 467 mm per year with 78.5% falling between June and September. The elevation, slope gradient, slope aspect and slope position of the site are 880–887 m, 16–18.5°, north, middle backslope, respectively. The parent material is Mesozoic intrusive and extrusive limestone [33]. The soil is a leached cinnamon soil with clay loam texture and is similar to a Typic Haplustalf [34]. The profiles are approximately 60 cm deep with 4–5 cm of forest floor (all surface organic horizons) in uncut stands. The dominant understory vegetation species are *Quercus mongolica* Fisch. Ex Ledeb, *Rhamnus davurica* Pall, *Corylus heterophylla* Fisch. Rx Trautv, *Vitex negundo* Linn. var. *heterophylla* (Franch) Rehd, and *Carex lanceolata* Boott.

The experiment was conducted in three pure Chinese pine plantations. Each stand had been thinned at different times so that the stands we sampled were thinned 40, 32, and 24 years previously, and the final trees per hectare ranged from 2125 to 2700. Prior to sampling (2008), tree measurements (diameter at breast height (DBH) and height) and site attributes (elevation, soil depth, pH, OM, and N) were measured (Table 1) for each thinning age. We use sampling season as a surrogate for soil temperature changes within the stand.

Table 1. Tree and site attributes since thinning.

Tree Attributes			Site Attributes				
Time Since Thinning (years)	**Height (m)**	**Diameter at Breast Height (cm)**	**Elevation (m)**	**Depth of Mineral Soil (cm)**	**pH**	**Organic Matter (g/kg)**	**Nitrogen - (g/kg)**
24	6.79	10.36	879	53	5.05 ± 0.18	16.4 ± 1.0	0.348 ± 0.085
32	8.03	12.40	882	53	5.44 ± 0.08	26.9 ± 1.2	0.372 ± 0.017
40	9.21	14.12	910	52	5.45 ± 0.04	29.3 ± 1.3	0.432 ± 0.023

Soil pH, OM, and N are values ± the standard error of the mean.

2.2. Soil Sampling and Physico-Chemical Analyses

We collected soil samples in spring (April), summer (July) and autumn (October) 2009 from nine 20 m × 20 m plots in each stand. Soil cores (the volume is 99.94 cm^3, 50.46 mm (diameter) × 50 mm (height)) were collected from two soil depths (0–10 cm, 10–20 cm). Before collecting the mineral soil cores, the forest floor was removed so it would not be mixed into the sample. Within each plot, six soil cores at each depth were collected along an S-shaped transect. Samples from each depth were composited into three replicate samples to decrease soil heterogeneity. All samples were passed through a 2 mm sieve to remove stones, particulate organic matter, and root fragments and then stored at 4 °C until processing (<24 hour). Soil pH was determined in a 2:1 (water:soil) paste, organic matter was determined by weight-loss after ignition at 550 °C for 5 hour, and N was determined by the Kjeldahl digestion method using H_2O_2 as the oxidant and a 0.5 g soil sample with 10 mL H_2SO_4 [35] followed by titration on a UDK 152 distillation and titration unit (VELP Scientifica, Usmate, Italy).

2.3. Extracellular Enzyme Activity Assays

Enzyme assays began within 48 hours of sample collection and activity was determined on air-dried samples. There were three repetitions of the samples. Soil invertase activity was measured using the sodium thiosulfate titrimetry method [36]. Invertase activity in soil suspension was measured after a 24 hours incubation at 38 °C. The other three extracellular enzymes (catalase, phenol oxidase, and urease) were measured using assay techniques modified from Guan [37]. Urease activity was measured by indophenol colorimetry (578 nm) using urea as the substrate; 5 g soil samples with 1 mL toluene were placed into 50 mL flasks. After 15 min, 10 mL of a 10% urea solution and 20 mL citrate buffer (pH = 6.7) were added and then incubated at 37 °C for 24 hours. After incubating, 3 mL of the filtrate were placed into a 50 mL flask and 4 mL sodium phenol solution and 3 mL sodium hypochlorite solution were added. Color reactions indicative of urease activity were measured after 20 min using a spectrophotometer at 578 nm. Urease activity was expressed as mg released NH_3-N by 1 g soil at 37 °C per day. Catalase activity was measured by adding hydrogen peroxide and determining the rate of hydrogen peroxide release over time [38]. Catalase activity was determined by titrating a standard solution of 0.02 mol/L $KMnO_4$ over 20 min in the presence of H_2SO_4 [38]. Activity is expressed as $KMnO_4$ g^{-1} dry sample. Phenol oxidase activity was measured using the iodine titrimetry method in which 1 g soil samples were mixed with 10 mL of 11% pyrogallol solution, placed into a 50 mL flask, and incubated at 30 °C for 2 hours. Once incubated, 4 mL of the filtrate was added to a 50 mL flask along with 4 mL sodium phenol and 3 mL sodium hypochlorite solutions. Finally, 4 mL citrate-phosphate buffer (pH = 4.5) and 35 mL diethyl ether were added. This mixture was placed on

an orbital shaker for 30 min and then filtered. Phenol oxidase activity was quantified by measuring light absorbance at 450 nm. A calibration blank for each enzyme was run with each set of samples. All determinations of enzyme activity were performed on triplicate subsamples taken from the soil composites. Fluorescence was measured using a microplate fluorometer with a 365 nm excitation and 450 nm emission filter.

2.4. Data Analysis

All enzyme assays are reported as means ± standard error. To ensure the data were homogenous, we used Levene's test [39] prior to analysis of variance. Data were not transformed as the homogeneity criteria were fulfilled. All statistical analyses were performed with the SPSS18.0 statistical package. We used a repeated measures ANOVA in SPSS 18.0 (IBM SPSS, Chicago, IL, USA) to evaluate statistical differences in time since thinning and season on soil enzyme activities (Table 2). For each enzyme, we averaged the 0–10 and 10–20 cm soil depth data for the analysis of season by time since thinning and used an average of all three seasons (spring, summer, and fall) for the depth by time since thinning analyses. We used a correlation to examine each enzyme and the differences of soil enzyme activity in each soil depth. Differences at $p < 0.05$ were considered statistically significant. When the effects of the dependent variables were significant, Duncan's Multiple Range test at the 5% level was used to compare means.

Table 2. *p*-values from the ANOVA for the effect of time since thinning, seasons (spring, summer and autumn), and their interaction on soil enzyme activities.

Sources of Variation	Invertase		Urease		Hydrogen Peroxidase		Phenol Oxidase	
	MS	***p***	**MS**	***p***	**MS**	***p***	**MS**	***p***
Time since thinning	0.743	0.031	0.028	0.037	2.093	0.001	0.007	0.0001
Season	2.199	0.044	0.028	0.036	2.920	0.002	0.003	0.0001
Time since thinning × Season	2.777	0.053	0.002	0.399	4.333	0.068	0.002	0.0001
Error	0.555		0.011		0.102		< 0.001	

×: The interaction between time since thinning and season. MS = mean square error.

3. Results

3.1. Seasonal Response of Soil Enzyme Activity to Pine after Thinning

3.1.1. Invertase Activity

Time since thinning had a significant effect on soil invertase activity within the same season (Figure 1). In all three seasons, invertase activity was significantly higher in the stands with the longest time since thinning (40 years), while there was no significant difference between stands thinned 24 or 32 years ago. Seasonal differences within each time since thinning were not always significant, but summer invertase levels were significantly higher than those in spring. All stands exhibited an increase in invertase activity in spring and summer with a drop in autumn.

3.1.2. Urease Activity

There was very little urease activity in these stands; particularly in spring and autumn. Peak activity occurred in the summer for all times since thinning (Figure 2). The stand thinned 40 years ago had significantly more urease activity than the stand thinned 32 years ago, but was not significantly different from the stand thinned 24 years ago.

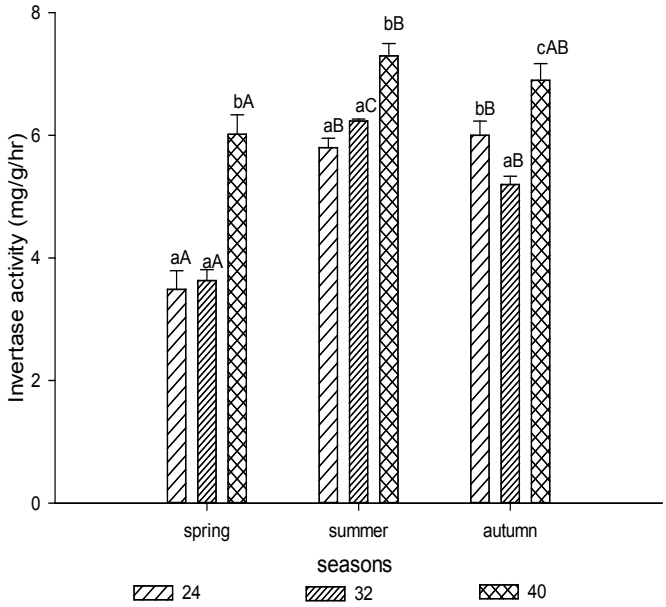

Figure 1. Influence of time since thinning (24, 32 and 40 years) and season on soil invertase activity. Capital letters indicate statistically significant differences among season within each time since thinning; lowercase letters indicate statistically significant differences among times since thinning within the same season. Bars indicate the standard error of the mean.

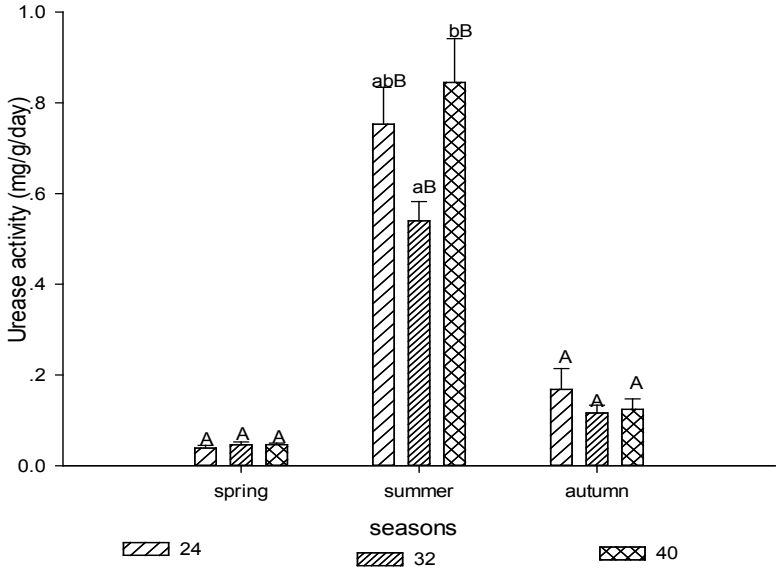

Figure 2. Change in urease activity during the growing season in stands with different times since thinning (24, 32 and 40 years). Within a time since thinning, capital letters indicate statistically significant differences among different seasons; lowercase letters indicate statistically significant differences among different times since thinning within the same season. Bars indicate the standard error of the mean.

3.1.3. Catalase Activity

Within each time since thinning, catalase levels generally declined or remained the same throughout the growing season (Figure 3); except in the longest time since thinning, where summer catalase activity was significantly greater. In the spring and autumn, catalase activity was significantly higher 24 years after thinning.

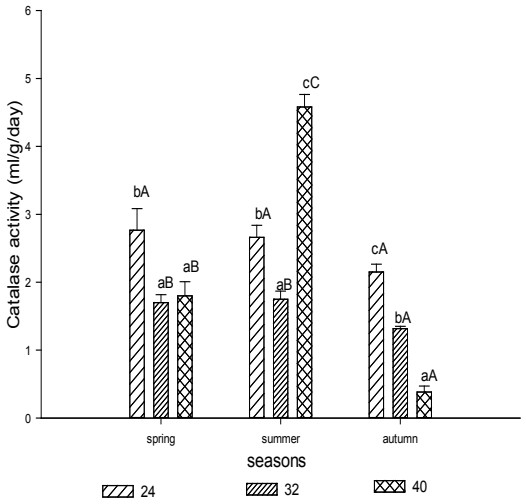

Figure 3. Influence of time since thinning (24, 32 and 40 years) and season on soil catalase activity. Capital letters indicate statistically significant differences among season within each time since thinning; lowercase letters indicate statistically significant differences among times since thinning within the same season. Bars indicate the standard error of the mean.

3.1.4. Phenol Oxidase Activity

Phenol oxidase activity was lowest in stands 24 and 40 years since thinning in both spring and autumn. The highest phenol oxidase activity in summer was found 32 years after thinning. This stand also had higher levels of phenol oxidase activity throughout the growing season (Figure 4).

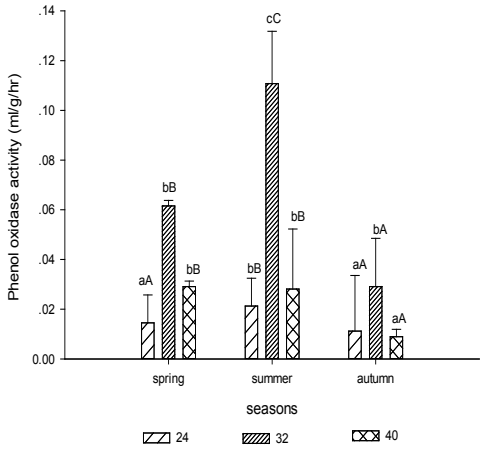

Figure 4. Change in phenol oxidase activity during the growing season in stands with different times since thinning (24, 32 and 40 years). Within each thinning age, capital letters indicate statistically significant differences among different seasons; lowercase letters indicate statistically significant differences among different times since thinning with the same season. Bars indicate the standard error of the mean.

3.2. Relationships Among Soil Enzyme Activity

Enzyme values were averaged for all stands and time since thinning before we conducted the correlation analysis. Urease was highly significantly correlated ($\alpha = 0.01$) with invertase activity when examined for all stands and seasons (Table 3). Catalase was also significantly ($\alpha = 0.05$) positively related to phenol oxidase activity. Both urease and invertase were negatively correlated with catalase and phenol oxidase, but this was not significant.

Table 3. Correlation among soil enzyme activity.

	Urease		Invertase		Catalase		Phenol Oxidase	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Urease			0.941 **	0.000	−0.140	0.766	−0.405	0.096
Invertase					−0.080	0.595	−0.261	0.058
Catalase							0.861 *	0.000
Phenol oxidase								

r: correlation coefficient; *: $\alpha = 0.05$; **: $\alpha = 0.01$.

3.3. Soil Enzymes at Different Soil Depths

Generally, soil enzyme activity was highest in the surface mineral (0–10 cm) compared to the deeper mineral soil (Table 4). In addition, invertase gradually decreased as time since thinning increased. Although not significantly different, catalase activity in the stand that was thinned 32 years ago was higher in the deep mineral soil (10–20 cm) compared with the surface soil. Phenol oxidase activity was much lower in both the surface and subsurface soil 40 years after thinning. Invertase, catalase, and phenol oxidase were all significantly higher in the surface mineral soil of the stand with the greatest time since thinning (40 years).

Table 4. Enzyme activity (± standard error) as influenced by time since thinning and mineral soil depth.

Time Since Thinning (years)	Depth (cm)	Invertase (mg/g/day)	Urease (mg/g/day)	Catalase (mL/g/day)	Phenol Oxidase (μL/g/h)
24	0–10	5.095 ± 0.138 **	0.319 ± 0.044 *	2.525 ± 0.145	35.057 ± 4.016
24	10–20	1.973 ± 0.101	0.135 ± 0.039	2.231 ± 0.243	32.818 ± 3.872
32	0–10	5.021 ± 0.383 **	0.234 ± 0.078 *	2.588 ± 0.084	70.807 ± 5.025
32	10–20	2.573 ± 0.243	0.169 ± 0.079	2.800 ± 0.191	66.680 ± 3.852
40	0–10	6.739 ± 0.231 *	0.339 ± 0.130	2.255 ± 0.144 *	15.743 ± 1.682 **
40	10–20	4.073 ± 0.160	0.248 ± 0.105	1.691 ± 0.207	7.542 ± 1.003

Asterisks indicate significant differences between soil depths within each time since thinning. *: $\alpha = 0.05$; **: $\alpha = 0.01$.

4. Discussion

The purpose of this study was to determine the relationships between time since thinning, season of sampling, and mineral soil depth on enzyme activity in a Chinese pine plantation. In our Chinese pine stands, we assumed that 20–40 years after thinning would be indicative of long-term soil enzyme changes in northern China.

For N-acquiring enzymes, urease activity varies significantly with time since thinning in Chinese pine plantations. Urease plays an important role in soil N cycling and utilization because it can hydrolyze urea to ammoniacal N [22]. Urease activity was highest 40 years after thinning (Figure 2). This is likely due to greater forest floor depth and N content in these older stands [40], which provides an N source for urease. These results are similar to those found by Barford [41] in which nitrification increased with stand age. In addition, in forest ecosystems, invertase catalyzes the hydrolysis of sucrose; one of the most abundant soluble sugars in plants [42]. Invertase is partially responsible for the breakdown of the forest floor material. Soil pH has little effect on soil invertase activity as long as the soil has a pH of 4–9 [43] and, although the pH in our stands increased slightly as time since thinning increased, the soil pH remained between 5 and 5.5. In addition to very low or high soil pH, the C:N ratio has also been shown to affect invertase activity [44,45]. Forty years after thinning allowed for the build-up of a thicker forest floor and likely increased mineral soil C cycling in the mineral soil. Immediately after thinning, the forest floor is reduced through decomposition processes, resulting in reduced C and N cycling [27]. Although we do not have OM or C contents for each soil depth

(0–20 cm depth only), we speculate that lower enzyme activity in the mineral soil of younger stands (24 and 32 years) may be related to lower C content [15]. In addition, we assumed the OM content of the mineral soil was approximately 48% C [46]. Using this value, we calculated the C:N ratios in the mineral soil as 22.6, 34.7, and 32.5 in our stands 24, 32, and 40 years after thinning, respectively. This increase in the C:N ratio has been noted after thinning in other stands [47] and likely changes soil N transformation rates. In addition, thinning may produce changes in the forest floor and soil microclimate as well as changes in N uptake resulting from the removal of tree basal area [48].

Phenol oxidases catalyze several reactions including the oxidation of manganese and iron [49] and the acquisition of C and N [31]. These enzymes can alter microbial communities because phenolic compounds are toxic. Some white rot (Basidiomycetes) and soft rot fungi (Ascomycetes) use intracellular phenol oxidases to synthesize protective compounds like melanin [27] while some organisms use them to degrade lignin [27,50]. In our study, both catalase and phenol oxidase activities were higher in the 24-year-old stand, and these enzymes decreased as time since thinning increased. These data support our hypothesis that soil enzyme activities will be different as time since thinning increases.

Soil microbial communities are subject to large seasonal variations in environmental conditions, such as temperature and moisture [51,52]. Moreover, the supply of nutrients also differs due to seasonal processes, such as the allocation of photosynthates to soil by roots of primary producers or the inputs of fresh litter and above- or belowground biomass production [53]. Seasonal changes in soil enzyme activity were also noted in other study areas. For example, soil phosphatase and β-glucosidase was higher in spring [54]; whereas peak invertase activity occurred in autumn [55]. The four soil enzymes we evaluated had peak activity in the summer when trees are actively growing. This also corresponds to generally higher soil temperatures and soil moisture since the monsoon season in our study area is from June-September. The summer sampling period, therefore, is the warmest and wettest of the three sampling periods. The monsoon climate promotes forest biomass production and microbial activity, resulting in higher enzyme production in the summer as compared to the other sample dates [53]. Both phenol oxidase and peroxidase activity (in general) has been shown to decline in the summer in other areas as a result of moisture limitations or carbon availability [49,56,57], but one study found no significant seasonal variation [6].

We attempted to reduce the spatial variation of the soil by compositing samples, but many soil enzymes are particularly variable on both the small (<1 m) and large (hectare) scale. However, where there is a gradient of pH, OM, or C, the microbial community is likely affected [58–60] and soil enzyme production is altered. For example, β-glucosidase activity varied significantly at both the individual tree scale (1.0 m) and the regional scale (55 ha) [61]. Except for catalase, which was lowest in autumn, the enzymes we studied were lowest during the spring sampling period. This difference could be caused by a dry, windy, spring conditions, resulting in both low soil temperature and moisture, which limits soil microorganisms and therefore, enzymatic activity. Autumn is the time when plants senesce and input litter onto the forest floor. Trees can affect both C and N availability for soil microbes via inputs by litterfall [62]. Labile C inputs in the form of new litterfall provide energy and enables microbes to degrade soil OM [63]. However, microbe activity may be confined to areas of new leaf litterfall [64]. These changes support our second hypothesis that yearly seasons affect soil enzyme activities.

Using cloning and sequencing of longer 16S rRNA gene fragments, microbial communities have been shown to change with increasing soil depth [64]. As a result, activities of microbial extracellular enzymes involved in C and N cycling and fungal laccases decline with increasing soil depth [64,65]. This decrease in microbial activity is similar to our results in which invertase and urease activities decreased with the increasing depth of soil (Table 4). The possible reason may be the lower levels of OM in the mineral soil in the two more recently thinned stands (Table 1) [55]. Further, subsurface soils often have a higher soil bulk density and corresponding lower soil porosity, resulting in poor soil aeration [66]. This likely reduces microbial species and quantity, ultimately decreasing soil enzyme

activity [67]. Root secretions are an important source of soil enzymes, and plant residue (litter and root structure) can increase enzyme production through decomposition [68,69]. In addition, soil macro- and microfauna may have an indirect role in soil enzyme activity. The results verify our hypothesis that soil depth influences soil enzyme activities. We also found that soil enzyme activity in the subsurface soil was lowest in the stands with the shortest time since thinning. Likely, the low canopy coverage of this young stand (3000 trees/ha as compared to 5600 or 3770 in the older stands) was less, thereby resulting in greater understory vegetation and root production [70]. Higher numbers of fine roots in the upper mineral soil horizons may have also contributed to increased enzyme activity [71,72].

5. Conclusions

Chinese pine plantation thinning operations are an important tool for maintaining forest health and increasing residual tree growth. We found that time since thinning is an important determinant of changes in soil enzymes. Overall increases in C:N ratios as a function of time since thinning can be a mechanism for altering enzyme activities over time, but not all soil enzymes respond to this change. For example, invertase activity increased as the time since thinning increased, while phenol oxidase activity increased and then decreased. Enzyme activities are likely the result of forest thinnings that alter microbial community structure through organic matter and nutrient inputs, disturbance frequency, and abiotic soil properties. Most enzyme activity that we measured was associated with the monsoon season in northern China; high summer activity and low spring activity. Changes in enzyme activity with soil depth was not unexpected. However, catalase activity increased slightly as depth increased and this should be further investigated. Our results in these Chinese pine stands indicate that changing enzyme activities may affect other site processes such as decomposition, carbon storage, and mineral soil organic matter content.

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Author Contributions: Ruiheng Lv, Yong Liu and Guolei Li conceived and designed the experiments; Ruiheng Lv performed the experiments; Weiwei Wang and Chen Xiao analyzed the data; Weiwei Wang wrote the paper. Deborah Page-Dumroese and Yong Liu guided manuscript writing and editing.

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