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SHORT COMMUNICATION

Variations in nitrogen, zinc, and sugar concentrations in Chinese fir seedlings grown on shrubland and plowed soils in response to arbuscular mycorrhizae-mediated process

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Abstract One-year-old seedlings of Chinese fir (*Cunninghamia lanceolata* (Lamb.) Hook) are not colonized with ectomycorrhizal (EM) fungi, but often colonized with arbuscular mycorrhizal (AM) fungi. The AM fungi could be important for nutrient acquisition in Chinese fir seedlings. Previous studies show that feedbacks between aboveground and belowground plant tissues play a fundamental role in controlling the interaction between plants and AM fungi. Our results indicate significant feedback in seedlings grown on shrubland soils, but not on plowed soils. The amounts of sugar in fir leaves in the shrubland soils were significantly lower than those in plowed soils. Leaf zinc (Zn) and nitrogen (N) concentrations were significantly higher in seedlings in shrubland soils than in plowed soils. In mycorrhizal seedlings growing in shrubland soils, leaf N:P ratios were significantly higher than those in plowed soils, likely because of enhanced N absorption through AM-mediated process. Leaf N:P ratios in seedlings grown on plowed soils were below the threshold levels, because of low metabolic activity of feedback in AM-mediated process. The results suggested that the presence of feedback between Chinese fir seedlings and AM fungi should be benefit in transplanting Chinese fir seedlings.

Keywords Arbuscular mycorrhizal fungi · Sugar · Zinc · Nitrogen · Feedback · ^{13}C natural abundance · Chinese fir seedlings

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Introduction

Chinese fir (*Cunninghamia lanceolata* (Lamb.) Hook) is usually established for timber production, with a rotation time of about 20–25 years (Zhang et al. 2004). Chinese fir has expanded rapidly since 1980s, accounting for 24% of total planted forest areas in China (Guo et al. 2009). Natural forests have been cleared and replaced by monoculture plantations of Chinese fir in southwest China. Traditional reforestation of Chinese fir involves planting of 1- to 2-year-old seedlings produced in nurseries on clear-cut and slash-and-burn sites that previously harbored native evergreen broad-leaved forests in southwest China. At present, most Chinese fir nurseries and plantations are in the second or third rotation on the same sites. Yield decline and site degradation (Zhang et al. 2004) have been ascribed primarily to a decline in soil C and nutrients largely because of slash burning during site preparation (Zhang et al. 2004). On plowed soils, the seedlings sometimes harbor diseases, and symptoms of N deficiency, such as the yellow discoloration of leaves.

The AM symbiosis is important for N and P nutrition of host (Smith and Read 1997), and depends on the host for sugars for the formation, maintenance, and function of the fungal structures (Zhu and Miller 2003; Ferrol and Pérez-Tienda 2009). Since sucrose is the major form of photoassimilates transported in higher plants, this sugar should play an important role in C delivery to the symbiosis (Ferrol and Pérez-Tienda 2009). The amount of C allocated to AM is estimated to range from 4% to 20% of a plant's total C budget (Smith and Read 1997). Arbuscular mycorrhiza may improve plant performance when plant is attacked by pathogens or by insect herbivores (Gange 2007). Arbuscular mycorrhiza colonization has been shown

to enhance plant growth and survival at low soil pH (Clark 1997), and its colonization plays an important role in plant NO_3^-–N uptake (Azcón et al. 2008). However, agricultural practices and clear-felling reduce the abundance and diversity of mycorrhizal fungi (Hedlund and Gormsen 2002; Brundrett 2009), and the destruction of mycorrhizae can lead to serious reforestation problems (Marshall 2000), especially for Chinese Fir. The role of mycorrhizal fungi associated with Chinese fir has not been recognized by farmers and managers. However, an attempt of scaling up and bridging microbial processes to the landscape and regional level can involve the selection of the right microbial indicators (Theuerl and Buscot 2010).

Arbuscular mycorrhiza can also take up Zn, and transfer it to the host plant (Cavagnaro et al. 2006), thereby enhancing plant Zn nutrition, particularly when it is present at low concentrations in soil (Davis et al. 2007). However, under high soil Zn concentrations, the formation of AM can also "protect" against the accumulation of Zn in plant tissues (Cavagnaro 2008). Arbuscular mycorrhiza may also influence nutrient availability via their effects on soil physiochemical and biological properties, such as pH (Li and Christie 2001), and nutrient cycling (Jackson et al. 2008). Plant N and P status can also have a significant effect on plant Zn nutrition (Marschner 1995), and the effects of N and P on AM may have consequences for plant Zn nutrition (Cavagnaro 2008). Despite the fact that numerous scientists have indicated that AM fungi promote N or immobile nutrient Zn of host plants independently, the interaction between N and Zn has rarely been investigated in Chinese fir mycorrhizal system.

In order to investigate the huge differences in Chinese fir seedling growth observed between shrub/grasslands and plowed soils, we divided nurseries into two groups: the first included land supporting shrub/grass after slash burning (4 sites) and sites with one rotation of plantations established after felling native forests (9 sites); the second was >2 rotation plantation sites (7 sites) and plowed sites, mainly paddy soil (4 sites). We measured colonization of mycorrhizal fungi and C isotope composition in seedlings grown on shrub/grass and deciduous forest after slash burning, which were optimum lands for growing seedlings, and grown on the plowed soils and >2 rotation plantation site. As a result of greater discrimination, the photosynthetic C sugars transferred from leaves to roots are more depleted in ^{13}C than nonphotosynthetic C derived from oxalacetate synthesized from phosphoenolpyruvate (PEP) reacting with HCO_3^- in roots, indicating indirectly the effects of AM fungi on plant C isotope composition since they mediate sugar transfer from leaf to root tissues. The aim was to determine whether Chinese fir seedlings grown on different land use histories responded differentially as determined by sugar and Zn concentrations, $\delta^{13}C$ values, and N:P, and to determine if N cycling in those plant–soil systems was tightly regulated by AM-mediated process. We hypothesized that despite the degree of colonization level by AM fungi, occurrence of feedbacks between plant tissues should enhance the ability of nutrient acquisition, such as Zn and N for Chinese fir seedlings.

Materials and methods

Research site

In this study, 24 nursery fields on slopes at different elevations above sea level were chosen as the sampling sites. The sites were in mountainous terrain, a transitional zone from low land to hills in the Guizhou province of southwest China. Sampling site elevation ranged from 280 to 1,510 m. Mean annual air temperatures decreased with altitude, and ranged from 18°C to 14.3°C. Most parts of the Guizhou Province have total annual precipitation >1,100 mm. Sampling sites were located in sandstone areas except for one site. The distribution of soil types varies with elevations; yellowish-red soils (Ultisols) were developed at altitudes below 600 m above sea level, and yellow soils (Ultisols) were developed at the altitudes of 600–1,500 m (Piao et al. 2001). The corresponding soil samples were taken at 0–25 cm depth with more than ten replicated cores for each soil sample to provide an indication of nutrient availability. The seedlings of Chinese fir were collected from nurseries in early January 2008 and 2009. The total biomass of each seedling was measured, and it was then separated into leaves, stems, and roots. Site selection aimed to capture a significant range of the variability in soil with usage histories. Fertilizers (15% N, 8% K_2O, 15% P_2O_5) were added in soil surface at all 24 sites before sowing seeds. Plowing was carried out in early spring, at a tillage depth of about 25 cm. Generally, the farmers sow seeds in February, and remove weeds at regular intervals. The nurseries on the shrub/grass and some of the one-rotation sites did not receive fertilizer additions of urea, but those from the plowed soils and >2-year rotation sites had urea added one or two times during one-grown period.

Laboratory analysis

Plant samples for laboratory analysis were dried for 48 h at 60°C and ground with a mortar and a pestle. Total C and N were determined with a CHNS autoanalyzer (PE 2400-ll). Metal elements and P were digested using nitric–perchloric acid digestion and analyzed by the atomic absorption spectrometry (AAS) and vanadomolybdate colorimetric methods, respectively. For soluble sugar determination, 0.25 g of air-dried plant material was collected in ten plots

and extracted four times with distilled water at 75°C, modified from the method of Chinnasamy and Bal (2003) because water was used instead of 80% of ethanol and the temperature of boiling water bath was 75°C. After each extraction, samples were filtered (Whatman No 42 filter paper), and the filtrates were used to determine soluble sugar colorimetrically through anthrone reaction (Piao et al. 2000). The concentration of available Zn and Fe of plant material was determined by ASS after diethylenetriamine-pentaacetic acid (DTPA) extraction (Lindsay and Norvell 1978). Standard soil analysis methods were used to measure soil-extractable Ca and Mg with 1 M NH_4OAC (Thomas 1982). The $\delta^{13}C$ values of plants were measured by combustion of 2-mg homogenized samples with CuO (1:50) at 850°C in a vacuum-combustion system. Carbon dioxide generated in the combustion tubes was separated by cryogenic distillation, collected in breakseals and analyzed on a mass spectrometer (MAT 252). The samples of Chinese fir seedlings were sent to the laboratory of China Agricultural University and examined for mycorrhizal colonization following the method of Gai et al. (2006). Briefly, the cleared roots with 10% KOH were acidified in lactic acid, and stained with Trypan blue, and examined for colonization level under a compound microscope. The data for $\delta^{13}C$ are expressed relative to the international standard PDB (as $\delta^{13}C$ ‰ (parts per thousand)). Each sample was analyzed in duplicate. The standard errors of C and N concentrations were always less than 0.01 g C kg^{-1}, and the standard deviations of the C isotope composition did not exceed 0.1‰. Differences between mean values of soil and plant nutrient concentrations were tested for their significance by using linear regression and variance analysis at 5% of probability.

Results

All of Chinese fir seedlings collected here were colonized by AM fungi, but not by EM fungi. Colonization levels with AM fungi varied from <10% to 74% with no differences between shrubland and plowed soils. According to field investigations, all of Chinese fir seedlings grown on shrubland soils did not harbor root rot and aboveground disease, but some of seedlings grown on plowed soils did, however, the cause the disease and N deficiency symptoms.

There were no significant differences in soil properties between shrubland and plowed soils. The average soil pH was 4.40±0.28, ranging from 4.08 to 4.96 in shrub soils, and 4.80±0.72, ranging from 4.18 to 5.14, with exception for one of 6.89, in plowed soil. The exchangeable Ca and Mg concentrations were 0.51±0.34 mg g^{-1} and 0.049±0.022 mg g^{-1} in shrub soils, and were 0.75±0.76 mg g^{-1} and 0.10±0.19 mg g^{-1} in plowed soils, respectively. The average ratio of soil C:N (13.0±2.3) in shrubland soils was similar to that in plowed soils (12.9±2.6). Available Zn extracted with DTPA solution (1.9±0.9 μg g^{-1}) in shrub soils was similar to that (1.7±0.9 μg g^{-1}) in plowed soils, and iron (Fe) concentration (0.080±0.029 mg g^{-1}) in shrub soils was also similar to that (0.073±0.042 mg g^{-1}) in plowed soils.

Most parameters of leaf tissues did not statistically differ between shrub and plowed soils (Table 1); however, the average concentrations of leaf sugar ranged from 18 to 166 mg g^{-1} in shrub soils and were significantly lower ranging from 56 to 164 mg g^{-1} in plowed soils. Leaf Zn ranging from 3 to 45 μg g^{-1} in shrub soils was significantly higher than values ranging from 3 to 26 μg g^{-1} in plowed soils. The leaf N:P ranging from 11.6 to 17.1 (mass) in shrubland soils was significantly higher than in plowed soils, where N:P ranged from 6.6 to 13.2 (mass; Table 1). Leaf C (%) ranging from 47.2% to 51.0% in shrub was higher than that in plowed soils, where leaf C ranged from 46.7% to 49.7%. Among root tissues, only Zn significantly differed between shrub and plow soils (Table 1).

Although there was no significant correlation between leaf sugar and biomass (height; $r^2=0.024$, $P>0.05$), biomass significantly decreased by decreasing root sugar ($r^2=0.385$, $P<0.01$; Fig. 1a). Leaf N was correlated positively with leaf P ($r^2=0.311$, $P<0.01$), and significantly correlated with leaf Zn ($r^2=0.413$, $P<0.001$; Fig. 1b). Leaf Zn significantly decreased with increasing leaf sugar ($r^2=0.175$, $P<0.05$), and leaf N also decreased with increasing leaf sugar ($r^2=0.212$, $P<0.05$; Fig. 1c). It seems that there was metabolic feedback between aboveground–belowground tissues in seedlings grown on shrubland soils. Leaf Zn was not significantly correlated with leaf P ($r^2=0.003$). Therefore, the significant correlation between leaf Zn and leaf N:P ($r^2=0.198$, $P<0.05$) was mainly determined by the variation of leaf N. Root N was not significantly correlated with biomass (height; $r^2=0.009$) and root sugar ($r^2=0.028$), respectively, whereas the root Zn was negatively and significantly correlated with biomass ($r^2=0.293$, $P<0.01$; Fig. 1d) and root sugar ($r^2=0.403$, $P<0.001$). This relationship was similar in fir seedlings on shrubland and plowed soils. This may suggest that the concentrations of sugar in the root did not necessarily reflect the degree to which sugar was available to the fungus.

The leaf biomass was always ^{13}C-depleted (−28.5±0.5‰) relative to the root tissues (−26.9±0.6‰), There was no difference of leaf $\delta^{13}C$ between seedlings from shrubland (−28.4±0.4‰) and plowed sites (−28.7±0.5‰), whereas root $\delta^{13}C$ (−26.7±0.5‰) in shrub was higher than that (−27.1±0.6‰) in plow.

Table 1 Some chemical properties of Chinese fir seedlings

Tissue		C (%)	$\delta^{13}C$ (‰)	Zn (μg g^{-1})	Sugar (mg g^{-1})	Ca (mg g^{-1})	Mg (mg g^{-1})	Fe (mg g^{-1})	N (mg g^{-1})	P (mg g^{-1})	N:P ratio (mass)
Leaf	Shrub	48.7 (0.9)	−28.4 (0.4)	25.1 (10.9)	85 (36)	7.5 (1.4)	1.13 (0.17)	0.11 (0.04)	20.8 (4.1)	1.5 (0.3)	14.2 (1.6)
	Plow	47.9 (0.7)	−28.7 (0.3)	15.7 (6.7)	124 (30)	7.5 (1.4)	1.20 (0.36)	0.11 (0.01)	17.4 (3.7)	1.7 (0.5)	10.8 (1.9)
	t test	$P<0.05$	$P>0.05$	$P<0.01$	$P<0.01$	$P>0.05$	$P>0.05$	$P>0.05$	$P>0.05$	$P>0.05$	$P<0.001$
Root	Shrub	45.8 (1.6)	−26.7 (0.5)	21.3 (11.3)	53.2 (22.1)	2.5 (0.4)	1.2 (0.4)	0.37 (0.14)	8.4 (2.1)	0.9 (0.2)	10.1 (2.5)
	Plow	46.4 (2.5)	−27.1 (0.6)	12.6 (9)	72.1 (30.7)	3.0 (0.5)	1.3 (0.5)	0.41 (0.23)	8.8 (4.0)	1.2 (0.5)	8.3 (5.8)
	t test	$P>0.05$	$P>0.05$	$P<0.05$	$P>0.05$	$P>0.05$	$P>0.05$	$P>0.05$	$P>0.05$	$P>0.05$	$P>0.05$

Discussion

Our results showed that there was no significant relationship between AM colonization levels and nutrient acquisitions in seedlings grown on both shrubland and plowed soils. But there were significant differences in the internal feedbacks in fir seedlings on shrubland and plowed soils. A key AM-mediated process is the transfer of photosynthate from host plant to AM fungi hyphae, and its residence time is normally days (Zhu and Miller 2003; Richardson et al. 2009). The fungi are obligate symbionts and cannot survive without photosynthate supply from plants (Smith et al. 2010). When less C is transferred from plants to fungus, this may be responsible for low concentrations of nutrients in seedling tissues grown on plowed soils. The extraradical hyphae of the AM fungi are a key component of AM symbiosis linking colonized roots with the soil matrix (Malcová et al. 2001), proliferating in the surrounding soil from which they absorb nutrients (Hawkins and George 2001). Our results suggested that colonized roots may play a more important role in exploiting N in plowed soils than in shrub/grassland soils. Soil disturbance and high soil compaction can destroy hyphal networks and reduce AM fungal populations (Drew et al. 2006), and mineral fertilization can reduce N uptake by AM fungi hyphae and mycorrhizal roots (Cheng et al. 2008). However, inorganic fertilizer was added in all of 24 sites onetime. It has been shown that urea does not affect on soil microbiological properties (Lupwayi et al. 2010).

At the sampling time, we observed new root emergence in shrubland sites, but rarely in plowed sites. The intra-radical colonization by mycorrhizal fungi is generally faster (peaking at 7 to 15 days) than that by nonmycorrhizal fungi and often more frequently in younger roots (Resendes et al. 2008). We found that even the seedlings showing N-

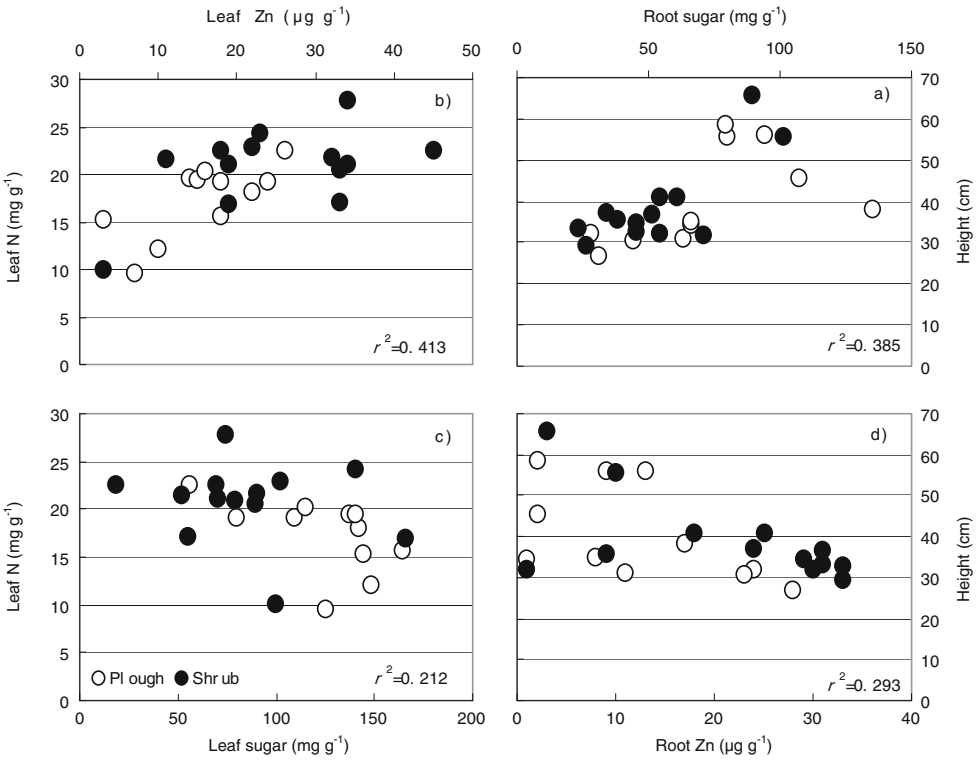

Fig. 1 Aboveground height as affected by root sugar (**a**), N content of leaf as affected by Zn (**b**) or sugar content of leaf (**c**), and aboveground height as affected by root Zn (**d**)

deficient symptoms (collected at January 2010) were also colonized by AM fungi (up to 36%) and developed root hair in sites after several rotations. Mycorrhizae are particularly important for nutrient uptake in plant species that do not develop dense root systems or do not develop root hairs (Jakobsen et al. 2005). It is apparent that roots grown on shrubland soils had higher metabolic activity than those grown on plowed soils, which should be responded to different soil conditions.

Aboveground–belowground feedbacks play a fundamental role in controlling the interaction between plants and soil organisms including AM fungi (Wardle et al. 2004). As already mentioned, the fungi efficiently take up inorganic ions from the soil and transfer them to the plant (Nygren et al. 2007), while the plant supplies the mycorrhizal fungi with products of photosynthesis (Baum et al. 2009), and the C transport is improved by root colonization by AM fungi. The low sugar in leaf tissues was correlated with higher leaf N and Zn in seedlings grown on shrubland soils in comparison with plowed soils, indicating that there was apparent aboveground–belowground feedback regulation. The lower leaf sugar in shrubland than in plowed soils should imply that more sugar was transferred to AM fungi-mediated processes in the former than in the latter soil, and extraradical hyphae of AM more efficiently absorb N and Zn in the former soils, and these nutrients were transferred from root to leaf tissues, resulting in increasing contents of leaf N and Zn in plants grown on shrubland soils. Previous study also shows that there is significantly less sucrose in both shoot and root in summer wheat with AM fungus compared to those without AM fungus (Hawkins and George 2001).

Threshold levels for nutrients in plant tissues have been suggested by Koerselman and Meuleman (1996), who indicate that N:P >16 indicates P limitation and N:P <13 shows N limitation. Characteristic ratios between N and P determine the basic stoichiometry for the plants, allowing us to predict their response to alterations of N and P availability (Schlesinger 2004). The seedlings grown on shrubland soils had higher N:P ratio values than threshold levels, except for 2 out of 13 sites, and seedlings on plowed soils had the N:P ratio values lower than threshold levels, except for 2 out of 11 sites. Therefore, most sites of plowed soils but not those of shrubland soils were under N-limited conditions. In the N-limited soils, like our nursery sites, the N delivery to the host plant from the mycorrhizal fungi depends on the C flow from the plant (Egerton-Warburton et al. 2007; Baum et al. 2009), and the transfer of plant C to mycorrhizal fungi is higher under conditions of low nutrient supply (Huygens et al. 2008).

It has long been thought that AM fungi do not contribute significantly to nitrate uptake by plants, but Azcón et al. (2008) demonstrated that NO_3^- appeared to be the best N source for AM plants in a neutral-alkaline soil and particularly under drought stress conditions. Chinese fir normally grows on low pH, moist, and fertile soils. Soil pH is known to have large effects on N cycling (Ste-Marie and Paré 1999). Despite the decrease in biological activities, net mineralization of N and P increases by decreasing pH (Kooijman and Hedenäs 2009). There are constraints on nitrification other than substrate supply in low soil pH, with N-limited conditions (Falkengren-Grerup et al. 2004). Net nitrate production in acidic forest floor is often very low or clearly absent, with prevalence of NH_4^+ (Ste-Marie and Paré 1999); even so, nitrate may still be a significant proportion of the N taken up by trees (George et al. 1999). By determining ^{15}N natural abundance, we have found that the increases of leaf N in seedlings grown on shrubland soils were associated with absorption of nitrate by Chinese fir (nonpublished data).

As already mentioned, AM can also increase uptake of Zn (Thompson 1990; Purakayastha and Chhonkar 2001; Cavagnaro et al. 2006). The contribution of mycorrhiza to total Zn uptake is estimated at approximately 50% in clover (Marschner 1995). Arbuscular mycorrhizal fungi may also influence nutrient availability via their effects on soil physiochemical properties, like soil pH (Li and Christie 2001), and nutrient cycling (Jackson et al. 2008). In addition, acidification of rhizosphere soil can improve Zn solubility and thus Zn availability (Cavagnaro 2008). Preferential solubilization of highly insoluble fractions, such as crystalline-bound and residual fractions of Zn, by AM fungi can occur in soil (Subramanian et al. 2009). Probably, the observed increase in Zn uptake by seedlings grown on shrubland soils was associated with higher Zn solubilization than in plowed soils.

The leaf biomass was always ^{13}C-depleted relative to the root, due to either fractionation processes or an increased proportion of PEPc fixed C in root tissues (Brandes et al. 2006). In our case, no differences of leaf $\delta^{13}C$ were found between shrubland and plowed sites. The root $\delta^{13}C$ in fir seedlings grown on shrubland soils was higher relative to that in plowed soils. Roots can take up HCO_3^- and consume it through the root PEPc. The selective incorporation is higher in roots than in leaves (Ford et al. 2007), and approximately 1% of total plant C can originate from root uptake of inorganic C (Ford et al. 2007; Rasmussen 2009). The fact that PEPc enzymatic activity is higher in NO_3^--fed than NH_4^+-fed plants could be explained by alkalinization of cellular sap caused by the reduction of the taken up nitrate in root cells (Pasqualini et al. 2001). However, the NH_4^+ nutrition can enhance the activity of PEPc in numerous plant species, and thus the bicarbonate assimilation in root cells (Pasqualini et al. 2001). In our case, the assimilations of HCO_3^- in the roots grown on shrubland soils may be higher than in plowed soils. In

addition, the difference of root $\delta^{13}C$ between shrubland and plowed soils could also be ascribed to difference in the amount of photosynthates delivered to the AM-infected roots.

Conclusion

To avoid parasitism, plants appear to develop feedback mechanisms to regulate the C drain to the fungal symbiont in relation to the nutrients given by fungus. Chinese fir seedlings are generally transplanted at early January every year. Because of key ecological functions performed by AM associations, low metabolic activity of feedback in AM-mediated process, and loss of mycorrhizal activity in degraded areas may limit the successful re-establishment of Chinese fir seedlings. At present, the information on mycorrhizal colonization in Chinese fir nursery and plantations is scarce, and an intensive sampling is required to resolve the mycorrhizal status of Chinese fir plant.

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