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Positive feedback of soil fungi, including arbuscular mycorrhizal fungi, to the invasive weed *Ageratina adenophora*: evidence from field studies

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Abstract: 【Background】Many alien plant species including *Ageratina adenophora* can establish a mutualistic symbiotic relationship with arbuscular mycorrhizal fungi (AMF) in their newly invaded habitats. Therefore, how these alien species invasions are mediated by mycorrhizal fungi urgently needs to be explored. 【Method】To explore the feedback of soil fungi, including AMF to the invasive plant *A. adenophora*, the soil chemical properties at different invasion stages (*A. adenophora* grown with native plants around [Partially invaded habitats]; and grown as its single species [Invaded habitats]) were measured, and then the feedback of soil fungi including AMF to *A. adenophora* were investigated in the field with fungicide treatment. 【Result】The chemical properties of soil were changed under *A. adenophora* invasion. Fungicide decreased the leaf area, carbon, nitrogen, phosphorus, and $\delta^{13}C$ content of *A. adenophora*. 【Conclusion and significance】Synthetic analysis found that soil fungi increased the carbon and the $\delta^{13}C$ content of *A. adenophora* leaves, but didn't increase the photosynthesis of *A. adenophora* in partially invaded habitats, indicating that the increased carbon and $\delta^{13}C$ content of *A. adenophora* leaves was not the result of photosynthesis but of some other mechanism. We supposed that carbon was transported from the soil or adjacent native weeds to *A. adenophora* through a mycelial network in partially invaded habitats. The changes in soil nutrients at different invasion phases may be one mechanism of *A. adenophora* invasion, and favors its establishment via fungi especially AMF, and transfer of carbon from soil or native plants stimulates its further spread.

Key words: *Ageratina adenophora*; arbuscular mycorrhizal fungi; carbon stable isotopes; invasive plant

丛枝菌根真菌在内的土壤真菌对入侵植物紫茎泽兰的正反馈:证据来自野外实验

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摘要: 【背景】包括紫茎泽兰在内的许多外来植物都能够与新入侵生境的丛枝菌根真菌(AMF)形成互利共生,因此菌根真菌如何调节外来植物种的入侵是当前亟待研究的问题。【方法】测定了紫茎泽兰入侵不同阶段(紫茎泽兰呈零星丛状分布于本地植物群落中[部分入侵生境]及紫茎泽兰单优群落形成期[入侵生境])的土壤化学性状,而后通过野外试验,采用杀

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真菌剂处理,研究了包括 AMF 在内的土壤真菌对紫茎泽兰入侵的反馈作用。【结果】紫茎泽兰入侵改变了土壤化学性状。施用杀真菌剂降低了紫茎泽兰叶面积、叶片碳、氮、磷、和 $\delta^{13}C$ 含量。【结论与意义】综合分析发现,在紫茎泽兰与本地植物混生群落中,土壤真菌能够增加紫茎泽兰叶片碳和 $\delta^{13}C$ 含量,但是不能提高紫茎泽兰的光合作用,表明碳和 $\delta^{13}C$ 含量的提高,不是光合作用的结果,而是通过其他机制实现的。因此可以得出,在部分入侵生境中,碳从土壤或临近植物经由菌丝网向紫茎泽兰转移。紫茎泽兰入侵不同阶段土壤养分的变化利于紫茎泽兰种群建立,同时利于紫茎泽兰借助真菌(尤其是 AMF)从土壤或临近植物转移碳,促进种群扩散,这可能是紫茎泽兰入侵的机制之一。

关键词: 紫茎泽兰; 丛枝菌根真菌; 碳稳定同位素; 入侵植物

Introduction

Plant invasion, being a multistage process like succession, is characterized by conspicuous spatio-temporal dynamics along the introduction – establishment – naturalization – invasion continuum (Callaway *et al.*, 2004; Shah *et al.*, 2009). Many alien plant species can establish a mutualistic symbiotic relationship with arbuscular mycorrhizal fungi (AMF) in their newly invaded habitats (Fumanal *et al.*, 2006). Therefore, with the overwhelming increase in invasive plants outside their original habitats, how are alien species invasions mediated by mycorrhizal fungi urgently needs to be explored.

The Crofton weed, *Ageratina adenophora* (Sprengel) King & Robinson (Synonym: *Eupatorium adenophorum* Sprengel) is a noxious perennial weed all over the world that is both allelopathic and toxic to livestock. Native to Mexico and Costa Rica, it has spread to over 30 countries in tropical and subtropical regions of the world. It was first introduced into Yunnan Province of China from Myanmar at the end of the 1940s; from there it rapidly spread to other southern and southwestern provinces of China including Sichuan, Guizhou, Guangxi and Chongqing (Jia, 2007). In Yunnan Province alone, *A. adenophora* has spread over 2,500,000 hectares, comprising 80% of the province. The weed has invaded woodlands, grasslands, roadsides and farmlands in these provinces, causing serious economic losses to agriculture, forestry, and livestock breeding (Xu *et al.*, 2006). It has out-competing native plants in many areas, rapidly forming monocultures that decrease biodiversity. Its invasion has especially endangered valuable plant resources, and has resulted in the loss of some ecosystem functions (Wan *et al.*, 2010). Previous studies had found that *A. adenophora* created a favorable soil environment to its own growth by changing the structure of the soil microbial community, including the arbuscular mycorrhizal fungi (AMF) (Li *et al.*, 2009; Niu *et al.*, 2007a, 2007b; Yu *et al.*, 2005).

With regard to the roles of mycorrhizal symbiosis in alien plant invasions, some hypotheses have been proposed, among which the resistance hypothesis (Kisa *et al.*, 2007; Mack, 1996) suggests that mycorrhizal symbiosis facilitates native plants in resisting alien plant invasion. However, increasing evidence has shown that mycorrhizal symbiosis exhibited positive feedback with alien plant invasion (Reinhart & Callaway, 2006; Richardson *et al.*, 2000), in which alien plant invasion inhibited the mutualistic symbiosis of mycorrhiza with native plants (Callaway *et al.*, 2008; Stinson *et al.*, 2006) and enhanced the competitive advantage of invading plants over native ones by virtue of mycorrhizal symbiosis (Carey *et al.*, 2004; Marler *et al.*, 1999; Reinhart & Callaway, 2004).

Currently, most studies on the feedback between AMF and alien plant invasions have been conducted as greenhouse experiments, with few being carried out as field investigations (Shah *et al.*, 2009). This study was conducted in the field to investigate the effect of soil fungi, including AMF, on the growth and the ability of *A. adenophora* to outcompete native plants.

Materials and methods

Study area

The study area was in Kunming, Yunnan Province (24°42′N, 102°52′E, altitude 1988 m) intensely invaded by *A. adenophora* (Li *et al.*, 2009; Niu *et al.*, 2007a, 2007b). This region has a subtropical

monsoon climate with the average temperature of 19 ~ 22 ℃ in summer and 6 ~ 8 ℃ in winter. The soil type of the test area is the typical red soil in southern China (Hapludult).

Experimental Sites

The experiment sits was in sparse coniferous and broad-leaf mixed forestry, and carried out in two type of communities, one community was still dominated by native plant species with clusters of *A. adenophora* spaced 2 ~ 3 m throughout (referred to as partially invaded habitats), while the other community was completely dominated by *A. adenophora* with a ten-year invasive history (referred to as invaded habitats), the invasion history was judged by observing the number of plant branchs, we found that the branch of *A. adenophora* increased one by year. For each type of community (partially invaded habitats and invaded habitats) we established five sites. Each site was about 200 m^2, and the distance between adjacent sites was 10 ~ 50 m.

Soil sample collection

To exam the differences in soil chemical characteristics between partially invaded habitats and invaded habitats, rhizosphere soil samples at 0 ~ 10 cm depth were collected for each location. To exam the differences in soil chemical characteristics during *A. adenophora* invasion, soil samples were collected from sites dominated by native plant species (referred to as uninvaded habitats). Five soil samples were collected from invaded habitats and uninvaded habitats respectively, and ten from partially invaded habitats. We considered that the different distance (from 2 m to 3 m) and the size (from 3 to 6 branch) of the clusters of *A. adenophora* in partially invaded habitats might result in difference of soil chemical characteristics more or less, so double soil samples were collected from this type of sites to eliminate the possible difference. The samples were air-dried, then sieved (2 mm mesh), and transferred to plastic bags.

Measurement of soil chemical characteristics

Soil pH, the concentration of soil organic carbon, total nitrogen, available phosphorus and available potassium were measured for each soil sample. Soil pH was measured using a glass electrode (WTW pH 340), soil organic carbon content was determined by the potassium dichromate method (Bao, 2000), total nitrogen was quantified using of the Kjeldahl method (Bao, 2000), available phosphorus was quantified using the colorimetric Mo-blue-method (Olsen & Sommers, 1982), and available potassium was extracted with 1 mol · L^{-1} ammonium acetate and then determined using the burnt-luminosity method (Bao, 2000).

Plot treatment and fungicide application

Five invaded habitats and partially invaded habitats was demarcated respectively, each site was demarcated into 10 plots. Each plot was 3 m × 3 m, at least 2 m from other plots, and 3 ~ 5 m away from trees. Five plots were randomly selected from the ten plots in each site, and had the fungicide benomyl, active ingredient: methyl 1-(butylcarbamoyl) benzimidazol-2-ylcarbamate, 0.05 g · m^{-2}, 1∶1000 dilution, applied as described by Callaway *et al.* (2004). These plots were termed the "fungicide treatment". As a control, tap water was applied to the other five plots in each site, and these plots were termed the "non-fungicide treatment". Fungicide application was repeated every two weeks, with four applications in total over the 64 days of the experiment.

Measurement of AMF colonization rate

To check the validity of fungicide treatment, for both partially invaded habitats and invaded habitats, Two hundred root segments were randomly selected from the roots of *A. adenophora* collected from each plot including 50 fungicide treatment and 50 non-fungicide treatment plots in invaded habitats and partially invaded habitats, and the colonization rate of AMF in the different treatments was measured. Fresh fibrous 1 cm root segments were soaked in 10% KOH for 25 min, rinsed with 2% hydrochloric acid for 5 min, then stained with 0.01% acid fuchsin-lactic acid-glycerin dye (acid fuchsin 0.1 g, lactic acid 875 mL, glycerin 63 mL, distilled water 300 mL) at 90 ℃ for 25 min. This was followed by diped in pure lactic

acid, after which the percentage of AMF root colonization was scored (http://invam.caf.wvu.edu/; Biermann & Linderman, 1981; Liu & Chen, 2007).

Measurement of photosynthesis and leaf area

Photosynthesis of *A. adenophora* plants was measured on morning after 64 days of the first fungicide application, the day when we measured photosynthesis was sunny and windless, a LI-6400 Portable Photosynthesis System was used. The controlled light intensity of the Photosynthesis System was 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and the CO_2 concentration was 400 $\mu mol \cdot mol^{-1}$, which determined by the light response curve and CO_2 response curve respectively. The photosynthesis rates were measured for the top 4th leaves from five plants in each plot. The temperature and atmospheric pressure were also measured at the same time. Subsequent measures of the photosynthesis rate and leaf area were determined using an LI-3000C Portable Leaf Area Meter.

Measurement of nitrogen, phosphorus, potassium, and stable carbon-isotope ($\delta^{13}C$) contents

One leaf samples were collected from per plot, and three of the 5 plots within a treatment were selected. After measuring the photosynthesis rate and leaf area, the upper new leaves (1st ~ 6th leaves) from each selected plant were harvested and brought to the laboratory, where they were cleaned by distilled water and dried at 60 ℃ for 68 h, and then ground using a carnelian mortar and passed through a 60-mesh sieve. After rapid digestion using the H_2SO_4-H_2O_2 method, the nitrogen, phosphorus, and potassium levels were measured with the Kjeldahl Nitrogen Analyzer, Mo-Sb calorimetry and the flame photometric method.

To measure the effect of fungicide on the carbon and stable carbon-isotope ($\delta^{13}C$) levels in leaves, a 0.010000 g sample was weighed and bundled using aluminum foil, and subjected to detection with a Thermo Finnigan Elementary Analyzer Flash EA1112 (Thermo Finnigan, USA). Reaction temperature was 95 ℃, 7×10^{-8} kPa of the vacuum, Cr_2O_3/Co_3O_4 oxidation furnace, 85 $mL \cdot min^{-1}$ of carrier-He flow rate, 80 kPa of Conflo-He pressure and 110 $mL \cdot min^{-1}$ of oxygen injection. The internationally used standard samples (HLY and UREA) were used in the determination of $\delta^{13}C$.

Statistical analyses

SPSS 13.0 software (SPSS Inc., Chicago, USA) was used for data analysis. Multiple comparison procedures for one-way ANOVA (one-factor analysis of variance) were used to analyze the differences between communities/sites and between the fungicide treatment and non-fungicide treatment. The means were separated using standard error.

Results

The changes in soil chemical properties in *A. adenophora* – invaded habitats at different invasion stages

The soil pH of invaded habitat was significantly lower than that of partially invaded habitats ($P < 0.001$) and invaded habitats ($P < 0.001$). While the organic carbon content of invaded habitats was 100% and 88.76% higher than that of partially invaded habitats ($P < 0.001$) and N ($P < 0.001$), respectively (Table 1). The total nitrogen content of invaded habitats was 75.7% and 61.4% higher than that of partially invaded habitats ($P < 0.001$) and uninvaded habitats ($P < 0.001$), respectively. The available potassium content of invaded habitats was 82.6% and 80.0% higher than that in partially invaded habitats ($P < 0.001$) and uninvaded habtitats ($P < 0.001$), respectively. Whereas, the available phosphorus content of invaded habitats was 27.2% and 29.2% ($P = 0.008$) lower than that of partially invaded habitats ($P = 0.007$) and uninvaded habitats ($P = 0.006$), respectively. No significant difference existed in the all above indicators of soil physico-chemical properties except soil pH between invaded habitats and partially invaded habitats ($P = 0.026$).

Validity of fungicide treatment

The colonization rate of AMF in fungicide-treated and non-treated plots was 19.7% and 93.6%, respectively, indicating that fungicide treatment significantly inhibited the infection of AMF on *A. adenophora*.

Effect of fungicide application on the photosynthesis of *A. adenophora* plants at different invasion stages

In non-fungicide treated plots, the photosynthesis rate of *A. adenophora* from invaded habitats was significantly higher than that from partially invaded habitats ($P=0.007$) (Fig. 1). However, in fungicide treated plots, no significant difference in photosynthesis of *A. adenophora* between invaded habitats and partially invaded habitats ($P=0.057$). While in invaded habitats, fungicide treatment had significant impact on the photosynthesis of *A. adenophora* ($P=0.004$), but not in partially invaded habitats ($P=0.676$).

Table 1 Soil chemical contents at different invasion stages of *A. adenophora*

Sample type	pH	Organic carbon ($g \cdot kg^{-1}$)	Total nitrogen ($g \cdot kg^{-1}$)	Available phosphorus ($mg \cdot kg^{-1}$)	Available potassium ($mg \cdot kg^{-1}$)
Uninvaded habitats	7.76 ±0.016a	35.81 ±1.71b	1.73 ±0.12b	25.52 ±1.84a	111.00 ±11.01b
Partially invaded habitats	7.69 ±0.018b	33.74 ±2.29b	1.59 ±0.10b	24.85 ±1.41a	109.40 ±6.01b
Invaded habitats	7.51 ±0.016c	67.60 ±5.25a	2.80 ±0.12a	18.08 ±0.80b	199.80 ±10.86a

Means in the same row followed different letters are significantly different at $P<0.05$.

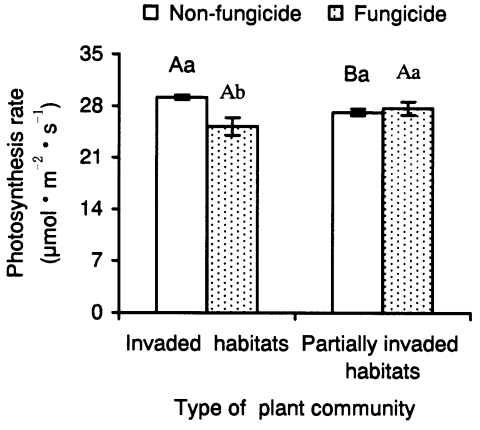

Fig. 1 Photosynthesis rate of *A. adenophora*

Bar heads with different capital letters indicate significant differences between invaded habitats and partially invaded habitats within the same fungicide treatment at $P<0.05$; bar heads with different lower case letters indicates significant differences between treatments within the same type of plant habitat at $P<0.05$.

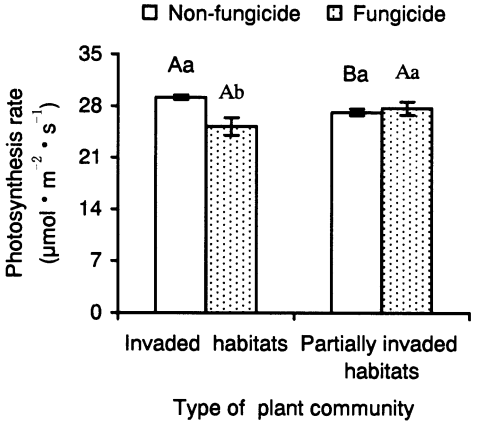

Fig. 2 Leaf area of *A. adenophora*

Bar heads with different capital letters indicate significant differences between invaded habitats and partially invaded habitats within the same fungicide treatment at $P<0.05$; bar heads with different lower case letters indicates significant differences between treatments within the same type of plant habitat at $P<0.05$.

Effect of fungicide treatment on *A. adenophora* leaf area at different invasion stages

No significant differences was found in the leaf area of *A. adenophora* between invaded habitats and partially invaded habitats in either non-treated ($P=0.224$) or treated ($P=0.089$) plots (Fig. 2). However, the leaf area of *A. adenophora* in both invaded habitats and partially invaded habitats, was significantly different between fungicide and non-fungicide treated plots (for invaded habitats $P=0.034$ and for partially invaded habitats, $P=0.043$), respectively.

Effect of fungicide application on carbon, nitrogen, phosphorus and potassium contents in *A. adenophora* leaves at different invasion stages

In non-fungicide treated plots, the carbon concentration in *A. adenophora* leaves from invaded habitats and partially invaded habitats, were not significantly different ($P=0.65$). The nitrogen and phosphorus content in *A. adenophora* leaves from invaded habitats was 15.6% and 32.7% higher than those from partially invaded habitats (for nitrogen: $P<0.001$ and for phosphorus: $P=0.005$), respectively. However, the potassium content in *A. adenophora* leaves from invaded habittats was significantly lower than that from partially invaded habitats ($P=0.03$). In fungicide-trea-

ted plots, the levels of carbon, nitrogen, phosphorus, and potassium in *A. adenophora* leaves from invaded habitats and partially invaded habitats were 463.1, 21.3, 1.1 and 21.5 g · kg^{-1}, which were 1.2% ($P = 0.02$), 14.1% ($P = 0.02$), 22.6% ($P < 0.001$) and 67.5% ($P = 0.007$) higher than that from partially invaded habitats, respectively. For both invaded habitats and partially invaded habitats, fungicide application resulted in a significant decrease in carbon (for invaded habitats: $P = 0.07$; and for partially invaded habitats: $P = 0.007$), nitrogen (for invaded habitats: $P = 0.02$; and for partially invaded habitats: $P = 0.004$), and phosphorus ($P < 0.001$) levels in *A. adenophora* leaves. However, fungicide application resulted in a significant decrease in potassium content in *A. adenophora* leaves from partially invaded habitats ($P < 0.001$), but didn't increase the potassium content in *A. adenophora* leaves from invaded habitats significantly ($P = 0.06$) (Fig. 3).

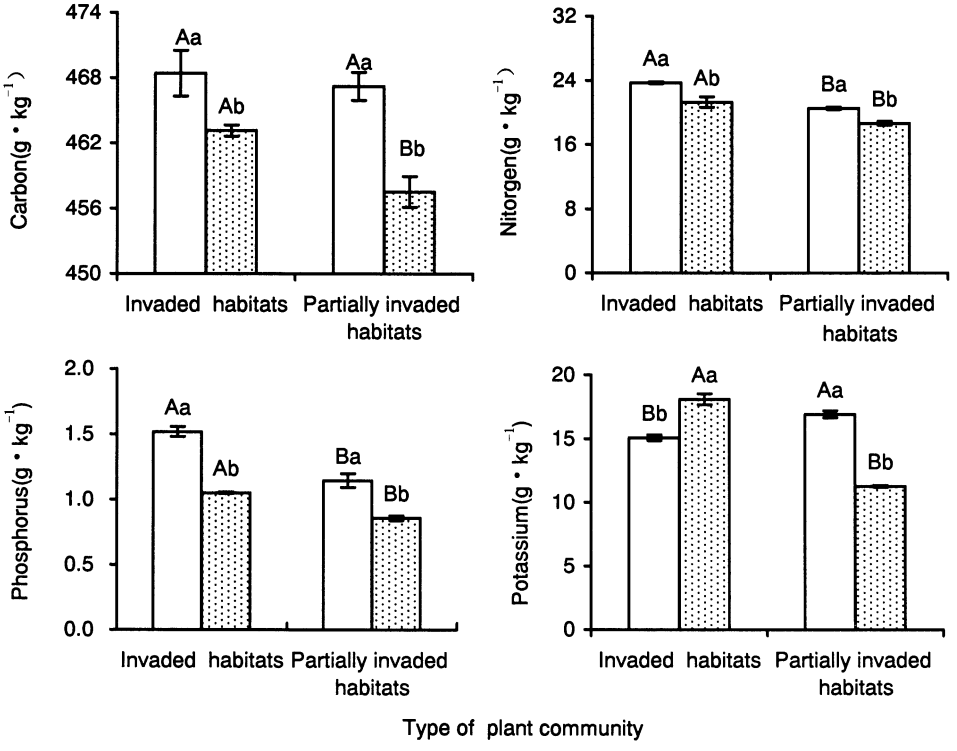

Fig. 3 The carbon, nitrogen, phosphorus (P), and potassium (K) contents in *A. adenophora* leaves

Bar heads with different capital letters indicate significant differences between invaded habitats and partially invaded habitats within the same fungicide treatment at $P < 0.05$; bar heads with different lower case letters indicates significant differences between treatments within the same type of plant habitat at $P < 0.05$.

Effect of fungicide application on stable carbon isotope content ($\delta^{13}C$) in *A. adenophora* leaves at different invasion stages

The stable carbon isotope content ($\delta^{13}C$) in *A. adenophora* leaves from partially invaded habitats in non-treated plots was significantly higher than that from non-treated plots in invaded habitats ($P = 0.001$) (Fig. 4). In fungicide-treated plots, no significant difference was found in $\delta^{13}C$ content between invaded habitats and partially invaded habitats ($P = 0.25$). The $\delta^{13}C$ values for both invaded habitats and partially invaded habitats in fungicide-treated plots was significantly lower than values for those same communities in non-treated plots (for invaded habitats: $P = 0.04$ and for partially invaded habitats: $P = 0.002$) respectively.

Effect of fungicide application on the carbon-to-nitrogen ratio in *A. adenophora* leaves at different invasion stages

The carbon-to-nitrogen ratios in *A. adenophora* leaves in non-fungicide treated plots from both invaded

habitats and partially invaded habitats were significantly lower than in fungicide treated plots (for invaded habitats A: $P=0.04$ and partially invaded habitats: $P=0.006$, respectively) (Fig. 5). The carbon-to-nitrogen ratio in *A. adenophora* leaves from invaded habitats was significantly lower than the ratio of leaves from partially invaded habitats, in both fungicide-treated and non-treated plots (for non-fungicide treatment: $df=1, 4$; $F=220.54$, $P<0.001$ and for fungicide treatment: $df=1, 4$; $F=14.65$, $P=0.02$), respectively.

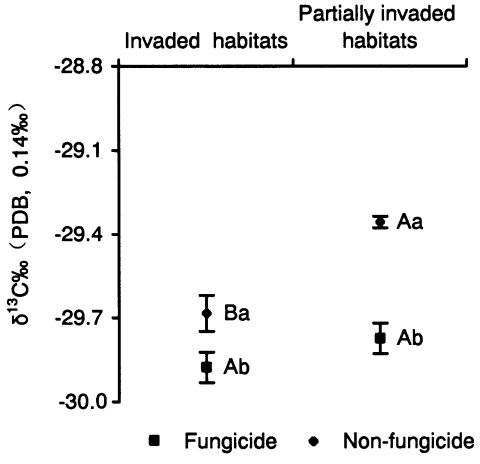

Fig. 4 Stable carbon isotope content ($\delta^{13}C$) in *A. adenophora* leaves

Bar heads with different capital letters indicate significant differences between invaded habitats and partially invaded habitats within the same fungicide treatment at $P<0.05$; bar heads with different lower case letters indicates significant differences between treatments within the same type of plant habitat at $P<0.05$.

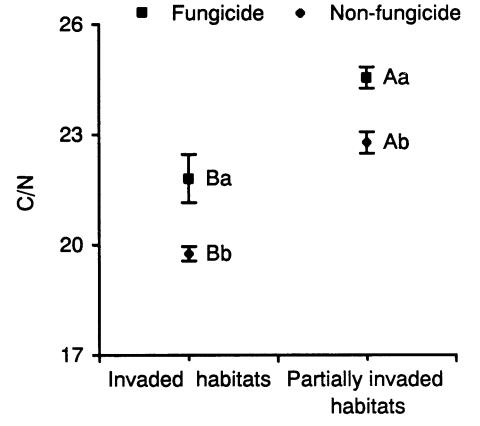

Fig. 5 The carbon to nitrogen ratio (C/N) of *A. adenophora*

Bar heads with different capital letters indicate significant differences between invaded habitats and partially invaded habitats within the same fungicide treatment at $P<0.05$; bar heads with different lower case letters indicates significant differences between treatments within the same type of plant habitat at $P<0.05$.

Discussion

The feedback of soil nutrients and AMF community on *A. adenophora* which influenced by its invasion

The invasion of *A. adenophora* disturbed the balance of soil nutrients, which in turn influenced the nutrient status of *A. adenophora*. Soil nutrients have a close relationship with the function of soil fungi, including AMF (Corkidi *et al.*, 2002), and invasive plants may change the structure and function of the microbial community through metabolic activity to alter the soil physical-chemical properties and soil ecology (Kourtev *et al.*, 2002; Mangla *et al.*, 2008; Shah *et al.*, 2008a, 2008b), thereby creating favorable conditions for invasion (Duda *et al.*, 2003).

The levels of organic carbon, total carbon, and available potassium in the rhizosphere soil of *A. adenophora* from invaded habitats were higher than those from partly invaded habitats, indicating that the invasive plant caused AMF to perform beneficial ecological soil-enriching functions, thereby creating a favorable nutrient feedback loop for *A. adenophora*, and AMF have a capability to degrade organic materials to inorganic components that plants can directly utilize, thereby promoting plant growth (Daehler, 2003; Hodge *et al.*, 2001; Leigh *et al.*, 2009).

The different types of feedback from soil AMF to *A. adenophora* at different invasion stages may result from the alteration of the AMF community structure and biodiversity caused by the *A. adenophora* invasion itself (Yu *et al.*, 2011, 2012), as Shah *et al.* (2010) and Wolfe & Klironomos (2005) supported.

Soil fungi transfer carbon for *A. adenophora*

AMF may influence the way in which *A. adenophora* (from partially invaded habitats) obtain carbon in other ways than photosynthesis. Carey *et al.* (2004) reported the similar conclusion. Evidence that *A. adenophora* obtained carbon from soil via AMF, or from the adjacent plants directly via carbon transfer was stable carbon isotope ($\delta^{13}C$) content in *A. adenophora* leaves of different treatments. Soil AMF in invaded habitats promoted carbon accumulation, and enhanced $\delta^{13}C$ con-

tent. The $\delta^{13}C$ content was −25.512‰ to −25.112‰ in red (Hapludult) soil (Qi *et al.*, 2009), which was higher than that in *A. adenophora* leaves, indicating that *A. adenophora* was exchanging carbon with soil. Therefore, *A. adenophora* grew in *A. adenophora* – dominated communities obtained carbon not only via photosynthesis, but also from soil organic substances.

The contribution of soil AMF in partially invaded habitats to the increase of carbon content was greater than that in invaded habitats. Therefore, soil AMF in partially invaded habitats transferred more carbon than that in invaded habitats. The contribution of soil AMF in partially invaded habitats to the increase of $\delta^{13}C$ content was greater than that in invaded habitats, suggesting that soil AMF in partially invaded habitats may transfer carbon with a higher proportion of $\delta^{13}C$, which is possibly from adjacent C_4 plants. Our field investigation showed that most native plants adjacent to *A. adenophora* – invaded habitats were C_4 plants (such as *Setaria faberii* Herrm and ferns). Carey *et al.* (2004) provided indirect evidence that AMF transferred carbon from the native plant *Festuca idahoensis* Elmer to *Centaurea cyanus* L. using stable isotope and physiological methods. Giovannetti *et al.* (2006) also supported the view that mycorrhiza regulated carbon transfer from native to alien plants, tipping the competitive balance toward alien plants.

Soil fungi, including AMF, mainly created feedback beneficial to *A. adenophora*. Soil fungi can also transfer carbon from adjacent native plants to *A. adenophora*. As the feedback favorable to *A. adenophora* established via a decrease in soil pH, an increase in organic carbon, total nitrogen and available potassium contents through soil fungi and the carbon transfer from soil or native plants via fungi, these may be important mechanisms of *A. adenophora* invasion.

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