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Study on associated nitrogen fixation of bamboo plants rhizosphere

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Abstract—Acetylene reduction activities of several bamboo species root have been investigated and determined. It is shown that with N_2-backfilling method, the maximum activity of two kinds of monopodia bamboo (*Phyllostachys heterocycla va. pubescens*, *Phyllostachys meyeri McClure*) can reach 3.9 and 2.1 nmol $C_2H_4\,g^{-1}$ dry root h^{-1} respectively; with the direct determining method, the maximum activities of four kinds of sympodia bamboo (*Dendrocalamus latiflorus Munro, Dendrocalamopsis beecheyana (Munro) Keng f., Bambusa textilis McClure* and *Bambusa chungii McClure*) can reach 20.5, 24.8, 10.8, 7.5 nmol $C_2H_4\,g^{-1}$ dry root h^{-1} respectively. When tested by enrichment culture method, for *Ph. pubescens* and *Ph. meyeri*, the maximum activities are 184 and 160 nmol $C_2H_4\,g^{-1}$ fresh root h^{-1}; for *D. latiflorus*, *D. beecheyana*, *B. textiles* and *B. chungii* species, the maximum activities are 978, 2743, 915, 397 nmol $C_2H_4\,g^{-1}$ fresh root h^{-1} respectively. The number of azotobacteria of four bamboo species at different rhizosphere position has been determined. It is shown that from non-rhizosphere soil to rhizosphere soil to root surface to root region the numbers of azotobacter increase sharply, and the rhizosphere effects of azotobacter are very obvious. Identification has been done to azotobacteria isolated from *Ph. pubescens* and *Ph. meyeri*. It is shown that in *Ph. pubescens* rhizosphere, the azoterbacteria mainly belong to *Bacillus polymyxa* and *Bacillus licheniformis*. In *Ph. meyeri* rhizosphere, it is *Klebsiella pneumoniae*. Using these strains (Nos 2, 12, 14, 7) to inoculating *Ph. pubescens* seedlings and *D. latiflorus* tissue culture plantlets, it is shown that inoculating associated nitrogen-fixing bacteria can promote the growth of the plantlets.

Key words: Bamboo; rhizosphere; azotobacter; associated nitrogen fixation; soil fertility.

INTRODUCTION

Since Döbereiner reported [4] that maize has the ability of associated nitrogen fixation with *Spirillum lipoferum*, scientists from many parts of the world have become interested in it. So far, many species of *Gramineae* crops have been

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determined, such as: maize (*Zea mays*), wheat (*Triticum aestivum*), Chinese sorghum (*Sorghum vulgare*), sugarcane (*Saccharum officinarum*), rice (*Oryza sativa*) and some pasture crops, and many azotobacteria have been isolated from the roots of these plants. Therefore it is considered that *Gramineae* plants possess the habit of associated nitrogen fixation with some azotobacteria, and many studies have been carried out [1, 2, 9].

Bamboo plants constitute an important forest resource in southern China, and even in the world. Bamboo has numerous species, wide distribution and covers a large area, and also belongs to the grass family. But it is not known if it possesses associated nitrogen fixation characteristics like other *Gramineae* species: we are not aware of any report to this effect. This paper, through determining root nitrogenase activity in several bamboo species; counting the number of azotobacteria in bamboo rhizosphere soil, root-surface, root-region; separating and identifying the main azotobacteria of bamboo rhizosphere; then inoculating the azotobacteria to *Phyllostachys heterocycla var pubescens* seedlings and *Dendrocalamus latiflorus Munro* tissue culture plantlets has produced some evidence of bamboo associated nitrogen fixation.

MATERIALS AND METHODS

Determining bamboo root nitrogenase activity

Material. Materials used were bamboo roots of *Phyllostachys heterocycla var. pubescens, Phyllostachys meyeri McClure, Dendrocalamus latiflorus Munro, Dendrocalamopsis beecheyana (Munro) Keng f., Bambusa textilis McClure* and *Bambusa chungii McClure. Ph. pubescens* grows in Hushan, Fuyang, Zhejiang, China. *Ph. meyeri* grows by the Fuchun River in Fuyang, Zhejiang, China. *D. latiflorus, D. beecheyana, B. textilis* and *B. chungii* grow in the bamboo garden of the Forest Institute of Guangxi and Guangdong, China.

Methods of determination. Three methods are used to determine bamboo root nitrogenase activity. They are the N_2-backfilling method, direct determining method, and enrichment culture method. All of these methods are base on the acetylene reduction method by gas chromatography. So the root nitrogenase activity in this paper is given as the ARA (acetylene reducing activity).

1. N_2-backfilling method [3]. Samples of root were collected from fields growing bamboo. Roots were harvested in the late afternoon to allow maximum accumulation of photosynthate. Bamboos were not removed from the soil, but with a hoe at bamboo base of stem to remove some roots with soil together. The material was then carried back to the laboratory using plastic bags. The roots together with soil were dipped immediately into water. As the soil becomes soft, it was washed off; vigorous rootlets (diameter smaller than 3 mm) were selected, using a filter to strain off surplus water; they were then cut into lengths

Table 1.
The main fertility characters of these soils

Locality	Soil name	Texture	pH	Organic matter (%)	Whole N (%)	Whole P (%)	Whole K (%)	Quick acting N (PPM)	Quick acting P (PPM)	Quick acting K (PPM)
Hushan, Fuyang	Red soil	Loamy clay soil	4.9	1.03	0.059	0.061	1.30	69	12.7	37.8
Bank of Fuchun River, Fuyang	Alluvial soil	Sandy loam soil	5.9	0.95	0.068	0.059	2.74	59	6.4	40.2
Nanning, Guangxi	Ortho laleritic red soil	Clay loam soil	5.0	1.57	0.107	0.082	1.53	87	14.2	49.6
Guangzhou, Guangdong	Alluvial soil	Sandy loam soil	4.8	1.34	0.078	0.076	2.95	76	11.5	46.2

of about 10 mm, weighing out 3 g of roots to place them into a bottle (30 ml in volume) with some water, and the bottle was closed with a rubber seal. Then the vials were evacuated three times and the atmosphere replaced by 4%(v/v) O_2 in N_2 and pre-incubated overnight at room temperature in this atmosphere. The overnight pre-incubation was adopted as a routine procedure to overcome a lag period prior to the onset of C_2H_2 reduction. C_2H_2 (10% by volume) was injected in the morning, then incubated at 32°C for 2 h and the C_2H_4 produced measured with a gas chromatography fitted with a 2 m × 3 mm Poropak N column at 100°C. All of these operations were conducted under sterile conditions.

2. Direct determining method. Because the N_2-backfilling method requires some apparatus, when the survey is in remote country, people cannot carry these instruments; also, when there are many samples, it is very time-consuming. In this case, after the bamboo roots were harvested and they were treated by the methods described above, we omitted part of the procedure — the vials were not evacuated and the atmosphere was not replaced by N_2. On the second day, C_2H_2 (10% by volume) was injected directly in the morning and incubated at 32°C for 2 h. Then the C_2H_4 produced was measured as above.
3. Enrichment culture method [5]. The materials used to prepare semi-solid N-free mineral media were: KH_2PO_4, 0.4 g; K_2HPO_4, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; $CaCl_2$, 0.02 g; $FeCl_3$, 0.01 g; $NaMoO_4 \cdot 2H_2O$, 0.002 g; Na malate, 5.0 g; agar, 1.75 g; H_2O, 1000 ml; pH 7.0. Root pieces (1 g, each about 5 mm length) were inserted into 2 ml of medium in a 6 ml serum bottle and incubated for 24 h at 32°C. Then the bottles were fitted with serum stoppers and C_2H_2

ARA >1 nmol C_2H_4 bottle^{-1} h^{-1}, it is recorded as '+', and when ARA <1 nmol C_2H_4 bottle^{-1} h^{-1}, it is recorded as '−'. Then use the MPN method [8] to estimate the number of azotobacteria in different rhizosphere positions.

Azotobacteria separation

Select the bottles described above under counting methods which have the highest ARA; take a loop of the liquid with azotobacteria from it; streak on solid medium plate to separate and purify the bacteria. The solid medium is the same as the above mentioned semi-solid medium in chemical composition, but agar in it is 20 g per 1000 ml. Inoculate the purified bacteria into serum containing 2 ml of the semi-solid medium, then determine ARA in the same way as above; select the bacteria with the highest ARA to research and identify.

Identification of azotobacteria

Identification of azotobacteria based on *Berey's Manual of Systematic Bacteriology* [7].

Inoculation of azotobacteria

Tested strains. Tested strains are No. 2 (*Bacillus licheniformis*), No. 12, No. 14 (*Bacillus polymyxa*) which are isolated from roots of *Ph. Pubescens*, and No. 7 [(*Klebsiella pneumoniae (Schroeter) Trevisan*)] from roots of *Ph. meyeri.*

Tested plants. Tested plants are *Ph. pubescens* (moso bamboo) seedlings and *D. latiflorus* tissue culture plantlets. The method of propagating *D. latiflorus* tissue culture plantlets is to, first, cut a new bamboo branch into small pieces, and sterilize with $HgCl_2$ solution. Then cultivate them *in vitro*, using hormones to induce them to generate buds in the node part prolifically. Then cut the bud at the base, cultivate the buds in another rooting medium again, and again let the bud generate roots *in vitro*. Take them out of the flask and plant in field. The purpose is to propagate plants rapidly. The plantlets in this experiment, coming from Guangdong Forest Institute of China, are about 5–8 cm high with a profusion of new roots and which have not been contaminated by air. The moso bamboo seedlings are prepared from seeds. After the seeds were sterilized by 0.15% $HgCl_2$ solution and then washed sterilized water, they were put into culture dishes that have been sterilized before and have filter and water in them. Then the dishes were kept at a temperature of 25°C to make the seeds germinate.

Medium. Medium for the trial was sand from Fuchun River. It was sterilized at 170°C for 4 h, and after cooling, it was put into plastic cups (300 g/cup), and 100 ml sterilized water added. It was then left to stand for 2 days to let the water permeate naturally.

Experiment design. There are four or five treated groups with one CK. They are No. 2 strain, No. 7 strain, No. 12 strain, No. 14 strain and mixed strain treatment groups with CK group. Each group has 15–16 repetitions. The statistical methods used are SSR (Duncan's new multiple range test) and PLSD (protected least significant difference test); before applying these tests, the data are subjected to variance analysis.

Inoculating. The azotobacteria, which have been purified and identified, were inoculated into Döbereiner N-free liquid medium respectively and cultivated for 24 hours. Then the bacteria cells were collected by centrifugation. 45 ml sterilized water and 5 ml Döbereiner N-free liqued medium was mixed to become 50 ml azotobacteria suspension (about 10^8 cells/ml) for inoculating. The same azotobacteria suspension was used for the CK group, but it had been sterilized by high temperature and pressure before inoculating. Mixed strain treatment involves equal volume mixing of four strain suspensions. Then just the germinating seeds are selected with tissue culture plantlets, they are put into a plastic cup that contains wet sand (one plant/per cup). 2 ml of the above-mentioned strain suspension was dropped on the roots and the sand nearby. Then the seeds and roots were covered by sad about 1–2 cm thick.

Plantlet cultivation. The plastic cups that contain wet sand for cultivating moso seedlings and *D. latiflorus* tissue culture plantlets are transparent and colorless. The wall of the cup is 1 mm thick. The diameters of the cup at the top and bottom are 80 mm and 52 mm respectively. There are 100 small holes (diameter 2 mm) on the bottom of the cup for leakage of the surplus water from the sand. The cup is covered and overlapped by other two cups at its upper end and bottom. Those two cups have a hole on the side, but the hole is covered by ventilation film (Commercial name, PARAFILM, American National Can Company.) so that it can ventilate, but cannot be penetrated by water or bacteria. Meanwhile, the cup overlapped at the bottom is smaller than the cup with wet sand, so it is just overlapped from the bottom to the middle of the cup that holds the sand so that it can contain the water penetrating from the sands into the culture cup. The joint parts of these three cups are also covered by ventilation film. All of these operations are carried out in a non-bacteria environment so that the seedlings are isolated from outside microbes and have only the inoculated bacteria. Then the seedlings are cultivated in artificial illumination for 14 h/day.

Testing. After cultivating for seven to eight months, several growth indexes of these plantlets are measured. Whole nitrogen is tested by the Kjeldahl method. The other parameters are measured by standard methods.

RESULTS AND DISCUSSION

Bamboo roots nitrogenase activity

There are three methods of measuring the six bamboos species roots nitrogenase activity. They are the N_2-backfilling method, the direct determining method and the enrichment culture methods. The ARA values measured by the N_2-backfilling method and the direct determining method are listed in Table 2.

In Table 2, when the activity of a sample >1 nmol $C_2H_4\,g^{-1}$ dry root h^{-1}, it is recorded as active. For monopodia bamboo species (*Ph. pubescens* and *Ph. meyeri*), among 48 samples, the highest can reach values of 3.9 and 2.1 nmol $C_2H_4\,g^{-1}$ dry root h^{-1} respectively. The ratio of active samples to the total is about 50%. In addition, because *Ph. pubescens* bamboo root is taken from sticky red earth, there is a thin soil layer adhering to the surface of the roots which it is very difficult to wash down. If the soil layer of the roots is not washed off, the nitrogenase activity of the root can rise to 9.9 nmol $C_2H_4\,g^{-1}$ dry root h^{-1}. For *Ph. meyeri* roots, because they are taken from sandy soil, there is no soil layer, so the nitrogenase activity is lower after it is washed with water. It is shown that the thin soil layer is made of soil and the excreting material of the root. Because there are many organic carbon sources and other nutrient factors, we believe that this is an active place for microbes. For sympodial bamboo species (*D. latiflorus*, *D. beecheyana*, *B. textilis* and *B. chungii*) the nitrogenase activity of roots are very high; they can be determined very easily by direct method, and the maximum activity of root may reach 20.5, 24.8, 10.8, 7.5 nmol $C_2H_4\,g^{-1}$ dry root h^{-1} respectively, the average activities are 4.8, 7.0, 2.0, 5.7 nmol $C_2H_4\,g^{-1}$ dry root h^{-1} respectively.

The ARA of six bamboo species root determined by enrichment culture method are listed in Table 3.

In Table 3, it is shown that for monopodial bamboo species (*Ph. pubescens* and *Ph. meyeri*), 90–95% of the root samples can be detected as having nitrogenase

Table 2.
ARA of six bamboo species roots (by N_2-back-filling method and direct determining method)

Method	Bamboo species		Sample number	Sample number with nitrogenase activity	Average activity (nmol C_2H_4 g^{-1} dry root h^{-1})	Maximum activity (nmol C_2H_4 g^{-1} dry root h^{-1})
N_2-back-filling method	Monopodia	*Ph. pubescens*	48	24		3.9
		Ph. meyeri	48	21		2.1
Direct determining method	Sympodia	*D. latiflorus*	48	40	4.8	20.5
		D. beecheyana	48	42	7.0	24.8
		B. textilis	48	32	2.0	10.8
		B. chungii	10	8	5.7	7.5

Table 3.
ARA of six bamboo species root (by N_2-backlilling method and direct determining method)

Method	Bamboo species		Sample number	Sample number with nitrogenase activity	Average activity (nmol C_2H_4 g^{-1} dry root h^{-1})	Maximum activity (nmol C_2H_4 g^{-1} dry root h^{-1})
Enrichment culture method	Monopodia	*Ph. pubescens*	47	45	43	184
		Ph. meyeri	30	27	40	160
	Sympodia	*D. latiflorus*	96	96	275	978
		D. beecheyana	96	96	431	2743
		B. textilis	96	96	169	915
		B. chungii	96	92	188	397

activity after they have been cultivated by the enrichment method; the average activities are 43, 40 nmol C_2H_4 g^{-1} fresh root h^{-1} respectively; the maximum activities are 184, 160 nmol C_2H_4 g^{-1} fresh root h^{-1} respectively. But for sympodial bamboo species (*D. latiflorus, D. beecheyana, B. textilis* and *B. chungii*), almost all of the roots can be determined as having nitrogenase activity. These samples are harvested at four periods (February, May, August, November), every time collecting 24 samples. In May and August, the nitrogenase activity is very high: the maximum can reach 978, 2743, 915, 397 nmol C_2H_4 g^{-1} fresh root h^{-1} respectively. In Feb and Nov, the nitrogenase activities are low. The average nitrogenase activities of bamboo species at four periods are 275, 431, 169, 188 nmol C_2H_4 g^{-1} fresh root h^{-1}, respectively.

Counting the number of azotobacteria in bamboo rhizosphere

Using C_2H_2-reducing-MPN (most probable number) method to count the number of azotobactria in different bamboo rhizosphere positions, the results are listed in Table 4.

In Table 4, the data show that the numbers of azotobacter are increased sharply from non-rhizosphere soil to rhizosphere soil to root surface to root region. This gives an obvious indication of the azotobacter distribution in bamboo rhizosphere. In four kinds of bamboo species, the most abundant in azotobacter is *D. beecheyana*.

Separating and identifying azotobacteria of bamboo rhizosphere

So far, eleven azotobacter strains have been isolated by us from sympodial bamboo rhizosphere and seventeen azotobacter strains have been isolated by us from monopodial bamboo rhizosphere. There are four strains with very high nitrogenase activity which have been selected by us as representative. The character of nitrogen fixing and the form of cells and colony has been researched and observed. Then they have been identified according to the data in *Bergey's Manual of Systematic*

Table 4.
Numbers of azotobacter in different rhizosphere positions (By C_2H_2-reducing-MPN method)

Bamboo species	Rhizosphere position	The numbers of azotobacter (cells/g dry soil or g dry root)	Rhizosphere effect at different rhizosphere position
Ph. pubescens	Non-rhizosphere soil	5.6×10^2	
	Rhizosphere soil	2.3×10^5	4.1×10^2
	Roots surface	4.3×10^6	7.7×10^3
	Roots region	4.3×10^6	7.7×10^3
D. latiflorus	Non-rhizosphere soil	3.2×10^3	
	Rhizosphere soil	9.0×10^5	2.8×10^2
	Roots surface	8.2×10^6	2.6×10^3
	Roots region	5.0×10^9	1.6×10^6
D. beecheyana	Non-rhizosphere soil	3.2×10^3	
	Rhizosphere soil	9.0×10^6	2.8×10^3
	Roots surface	3.6×10^7	1.1×10^4
	Roots region	2.1×10^9	6.6×10^5
B. textilis	Non-rhizosphere soil	1.4×10^2	
	Rhizosphere soil	1.7×10^2	1.2
	Roots surface	3.6×10^5	2.6×10^3
	Roots region	5.0×10^5	3.6×10^3

Table 5.
Four azotobacter strains sifted from bamboo rhizosphere

Strains	7#	2#	12#	14#
Origin	*Ph. meyeri*	*Ph. pubescens*	*Ph. pubescens*	*Ph. pubescens*
Species name	*Klebsiella pneumoniae*	*Bacillus licheniformis*	*Bacillus polymyxa*	*Bacillus polymyxa*

Bacteriology. The results of identification of these four main strains are listed in Table 5.

Azotobacteria inoculation

Using four strains (Table 5: Nos 7, 2, 12, 14) to inoculate *Ph. pubescens* seedlings and *D. latiflorus* tissue culture plantlets, the results are as below.

Effect of inoculating azoterbacteria to Ph. pubescens seedlings growth. After measuring several growth indexes and testing by variance analysis, it is shown that the average number differences of three growth indexes (see below) reach significant level. Then, further analysis yields the following results.

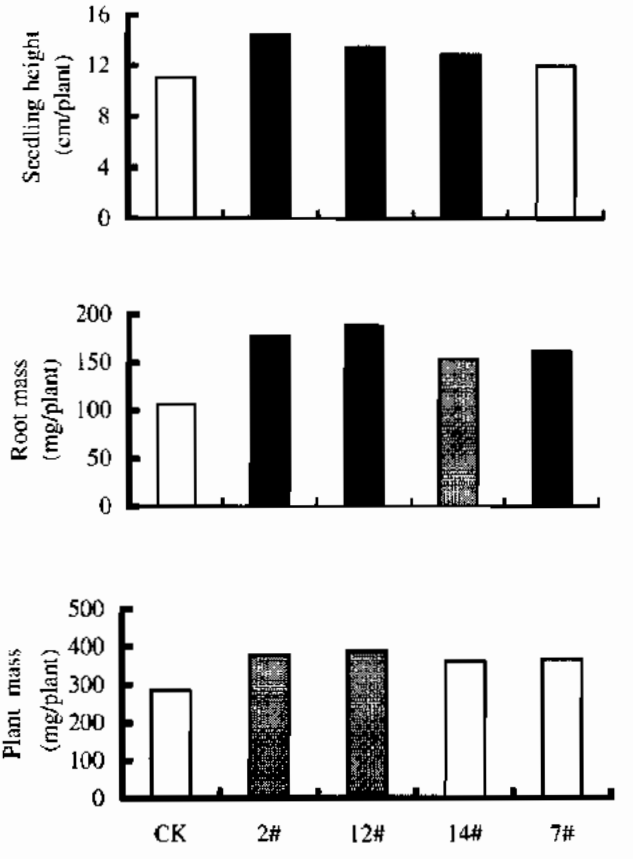

Figure 1. The effect of inoculating azotobacters on seedling growth of *Ph. pubescens*. The white posts indicate there are no diferences betweeen treatments and CK. The gray posts indicate the differences between treatments and CK are significant at $t_{0.05}$ level. The black posts indicate the differences between treatments and CK are remarkable significant at $t_{0.01}$ level.

Plant fresh weight. From Fig. 1, it is shown that after inoculating azoterbacteria to *Ph. pubescens* seedlings, all inoculating treatment groups have some increment than CK group. The increasing rate are by about 28.9%~36.9%. Using the SSR test, the differences between CK and No. 2 and No. 12 strain treatment groups reach significance level.

Root fresh weight (see Fig. 1). From the SSR test, it is shown that the differences between CK and four treatment groups are significant. Among them, Nos 2, 12, and 7 strain treatments show a remarkably high significance level and No. 14 strain treatment is also up to significance level. Therefore, inoculating with azotobacteria can promote the growth of roots. Comparing with CK, it is increased by about 44.1–76.9%. Those results are in keeping with some previous reports [10]. It is considered that inoculating associated nitrogen-fixing bacteria can change the form of roots, stimulate the growth of lateral root and increase the number of root hairs.

Height growth of seedling. From Fig. 1, it is shown that the seedlings of inoculating treatment groups are higher than the seedlings of CK group. The growth rates are increased by about 8–30%. From the SSR test, the differences between the CK group and Nos 2, 12, and 14 strain treatment groups show a remarkable significance level.

Effect of inoculating azoterbacteria to D. latiflorus *tissue culture plantlets growth.* After measuring several growth indexes and testing by variance analysis, it is shown that the average number differences of three growth indexes (see below) reach a remarkable significance level. Again, further analysis produced the following results.

Dry mass of plant. From Fig. 2, it is shown that all inoculating treatment groups have some increment compared with the CK group. Through the PLSD test, the differences between CK with Nos 14 and 7, and mixed strains treatment groups reach a remarkable significance level, with increase rates up to 150.7–220.8%. But Nos 2 and 12 strain inoculation plants are non-significant.

Dry mass of above ground part. From Fig. 2, it is shown that all inoculating treatment groups have some increment compared with the CK group. Through the PLSD test, the differences between CK with Nos 14 and 7, and mixed strains treatment groups reach a remarkable significance level, with increase rates up to 169.3–242.7%. But Nos 2 and 12 strain inoculation varieties are non-significant.

Dry mass of ground part. From Fig. 2, it is shown that all inoculating treatment groups have some increment compared with the CK group. Through the PLSD test, the differences between CK with Nos 14 and 7, and mixed strains treatment groups reach a remarkable significance level, with an increase rate up to 135.7–203.5%. But Nos 2 and 12 strain inoculation varieties are non-significant.

Effect of inoculating azotobacteria to plantlet survival rate and plant nitrogen concentration

Plantlet survival rate. After inoculating, the plantlets were cultivated for seven to eight months, then the survival rates were observed. If the plantlet withered and became completely yellow, it was recorded as dead. Any plantlet that had one or more green leaves was recorded as alive. From Fig. 3, it is found that comparing with CK, the survival rates of treatment groups are higher. The increasing rates for *Ph. pubescens* seedlings are by about 14.8–18.5%; for *D. latiflorus* tissue culture plantlets are by about 37.5–56.2%. The plantlets that resulted from the treatment groups also showed better growth than the CK plants. The plantlets of treatment

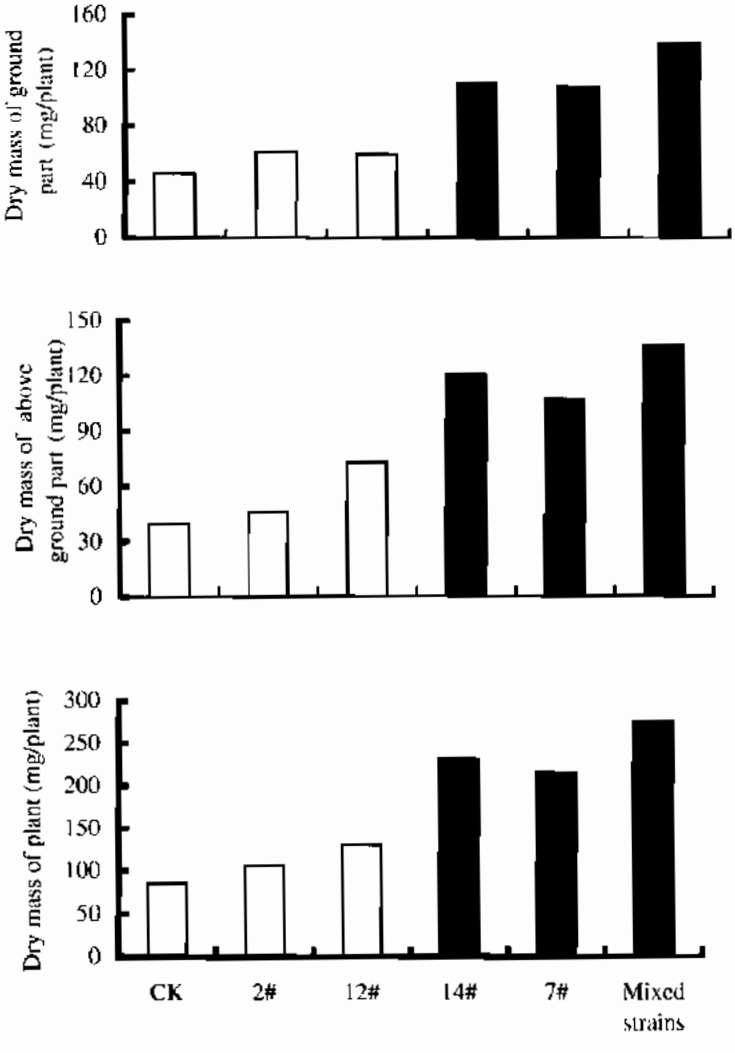

Figure 2. The effect of innoculating azoterbacters on *D. latiflorus* tissue culture plantlets growth. The black posts indicate the differences between treatments and CK are remarkable significant at $t_{(0.01)}$ level. The white posts indicate the differences between treatments and CK are non-significant.

groups were green and vigorous while the plantlets of the CK group were weaker, with some or all of the leaves yellow or dead.

Plant nitrogen concentration. From Fig. 3, it is shown that after inoculating nitrogen-fixing bacteria, plant nitrogen concentration is increased significantly. Comparing with CK, for *Ph. pubescens* seedlings the increment percentages are about 22.0–38.1%; for *D. latiflorus* tissue culture plantlets the increment percentages are about 9.8–53.7%.

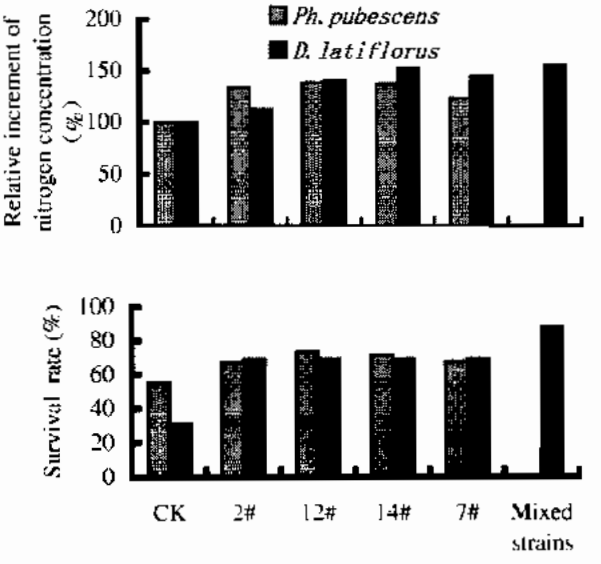

Figure 3. Effect of inoculating azotobacters on nitrogen concentration and survival rate of bamboo plantlet.

CONCLUSION

Through investigation and study of several bamboo species, it has been shown that there is associated nitrogen fixation in bamboo rhizosphere; furthermore, inoculating associated nitrogen fixing bacteria can promote bamboo plantlet growth.

Bamboo forest is an important part of the forest ecological system with higher management in China. In order to maintain a high fertility in bamboo forest soil and a high productivity, people have had to apply fertilizers, especially chemical fertilizers. But there are some problems from misuse of chemical fertilizer, especially for environmental protection.

Biological nitrogen fixation is an important way to input nitrogen from the atmosphere to the forest ecological system. It is the most environmentally secure and most economic way. So now scientists are becoming more and more interested in it, but until today, people have not paid attention to associated nitrogen fixation of bamboo. This research brings a hope that we may resolve the bamboo nitrogen supply finally by biological nitrogen fixation.

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