

## The effect of applying different amounts of nitrogen to tobacco seedlings on their growth and physiological characteristics

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### Abstract

In this study, we sought to elucidate how under- and over-application of fertilizers, which results often from erroneous calculation of the amount of fertilizer applied, affects tobacco seedling growth. Specifically, the effect of application of different amounts of nitrogen on the growth and physiological characteristics of seedlings of flue-cured tobacco K326 was investigated. Nitrogen treatment was done at four levels: 6 mg/plant (T<sub>1</sub>), 11 mg/plant (T<sub>2</sub>), 16 mg/plant (T<sub>3</sub>), and 21 mg/plant (T<sub>4</sub>). The root activity, enzyme activity, chlorophyll, photosynthetic performance, and root morphology were measured at 39, 45, 51, 57, and 63 days after seeding. Root activity exhibited a declining trend with increase in the amount of nitrogen applied at the late growth stage of tobacco seeding. Root activity decreased from 115.24  $\mu\text{g g}^{-1} \text{h}^{-1}$  (T<sub>1</sub>) to 109.65  $\mu\text{g g}^{-1} \text{h}^{-1}$  (T<sub>2</sub>), then to 85.07  $\mu\text{g g}^{-1} \text{h}^{-1}$  (T<sub>3</sub>), and finally to 76.86  $\mu\text{g g}^{-1} \text{h}^{-1}$  (T<sub>4</sub>) at 63 days after seeding. Moreover, nitrogen application also significantly increased the activity of resistance-related peroxidase, polyphenol oxidase, and phenylalanine, and enhanced the resistance of tobacco seedlings to pathogens. Applying the appropriate amount of nitrogen (6–11 mg/plant) could significantly increase the photosynthetic rate of tobacco seedling leaves, intercellular CO<sub>2</sub> content, transpiration rate, and water use efficiency. The amount of nitrogen most suitable for growth of tobacco seedlings with strong roots and resistance on a floating bed was 6-11 mg/plant.

**Keywords:** Nitrogen application; Tobacco seedling; Growth and development; Physiological characteristics.

**Abbreviation:** PPO, polyphenol oxidase; POD, peroxidase; PAL, phenylalanine ammonia lyase; P<sub>n</sub>, net photosynthetic rate; G<sub>s</sub>, stomatal conductance; C<sub>i</sub>, intercellular CO<sub>2</sub> concentration; T<sub>r</sub>, transpiration rate; WUE, water use efficiency.

### Introduction

Tobacco (*Nicotiana tabacum* L.), particularly flue-cured tobacco, is one of the most economically important agricultural crops, and has an important role in improving economic conditions in rural China. Adopting suitable modern technologies to produce healthy, high quality seedlings at a high yield is crucial for generating economic benefits from tobacco. Seedling production via the floating-bed system is widely applied for producing tobacco transplants in China (Ma et al., 2003). This system allows the cultivation of robust tobacco seedlings with a well-developed root system, strong resistance, and consistency (Yang et al., 1997). In addition, the floating-bed system eliminates soil-borne diseases that occur during the seedling stage, and minimizes production cost and seedling age (Shi and Wang, 2001). Therefore, the floating-bed system is particularly suitable for specialized and large-scale tobacco production (Lv and Yi, 2006). Currently, the floating-bed technique is the main method for growing tobacco seedlings in China (Bu et al., 2008) since it allows improvements in the quality of tobacco seedlings, pest control, and reduction of labor requirements for tobacco farmers (Liu, 2000). However, many problems have been encountered in the practical application of floating-bed techniques, such as salting-out of the substrate, spiral roots, green algae, and viruses (Peng et al., 2010), which are related to the use of inadequate

technologies for immature seedling (Fan et al., 2013), fertilizing (Yang et al., 2006), leaf removal (Zeng, et al., 2008; Liu et al., 2003), and acclimatization (Wu and Wu, 2012).

The fertilizing technology used determines the success of seedling production by the floating system. Among the mineral nutrients required for the growth and development of tobacco seedlings, nitrogen fertilizer is the most important. Many studies have been carried out on the growth of tobacco seedlings using the floating system. However, these studies were conducted in the standard density of nutrients (Jones, 1993; Li, et al., 2003; Xi et al., 2008). In addition, given the different amounts of nutrients in different nutrition pools, the amount of fertilizer used may be excessive or fall short, making it difficult to guarantee fertilization. Currently, arbitrary nitrogen fertilizer application is the most important problem in the seedling production process, and has a large effect on the quality of tobacco seedlings. Here we investigated the standard amount of fertilizer applied per tobacco plant instead of unit area, aiming to determine the amount of nitrogen fertilizer needed to grown tobacco seedlings in floating beds and to provide a scientific basis for nitrogen fertilizer application in floating-bed tobacco seedlings.

## Results

### *Influence of N on root morphology*

The influence of applying different amounts of nitrogen on root morphology is shown in Figure 2. Except for the samples at 39 days, T<sub>1</sub> and T<sub>2</sub> had the longest roots, with slight difference in various periods, whereas the roots in T<sub>3</sub> showed a decreasing trend. The diameter of roots processed with high nitrogen levels was the smallest initially, but gradually increased and stayed at a high level. The diameter of roots processed with low nitrogen levels was the largest initially, but were maintained at a low level. The root surface area of T<sub>1</sub> was small at the beginning and gradually increased to the highest level. The root surface areas of the other three groups changed, with T<sub>4</sub> having the lowest values in later determinations. The changes in root volume in T<sub>1</sub> and T<sub>2</sub> were smaller. The root volume in T<sub>3</sub> was the largest in later determinations. The root volume in T<sub>4</sub> was the smallest both at the beginning and the end of determination.

### *Influence of N on the activity of tobacco seedling roots*

The effect of applying different amounts of nitrogen on tobacco seedling root activity is shown in Figure 3. A slight difference was observed in the root activity of tobacco seedlings being processed at the beginning of the determination. Furthermore, with the application of increasing amounts of nitrogen, the activity of tobacco seedling roots showed a decreasing trend. In the middle of determination (51 days), the activity of the roots in T<sub>1</sub> was 2.28 times that of T<sub>4</sub>, 1.79 times that of T<sub>3</sub>, and 1.49 times that of T<sub>2</sub>. The reason for the high root activity in T<sub>1</sub> may be the low amount of nitrogen fertilizer applied and thus the low nitrogen content in the nutrient solution. Roots continued to grow and elongate, and maintained relatively strong vitality for obtaining nitrogen nutrients.

### *Influence on chlorophyll levels*

The changes in the chlorophyll levels of tobacco seedling leaves under different amounts of nitrogen are shown in Figure 4. An increasing trend was observed in the chlorophyll content with increasing amounts of nitrogen applied; except for T<sub>1</sub>, the chlorophyll contents in the remaining processes gradually increased with increasing seedling age. The chlorophyll content in T<sub>1</sub> was the highest in seedlings at 57 days and then decreased gradually. This decrease may be attributed to the growth of tobacco seedlings causing a gradual depletion of nitrogen. The difference in the chlorophyll contents between T<sub>2</sub> and T<sub>3</sub> tended to decrease gradually and was lowest at 63 days (the content was up to 37.4  $\mu\text{g L}^{-1}$  and 37.6  $\mu\text{g L}^{-1}$ ). The chlorophyll content in high-nitrogen processes was the highest and increased gradually as the seedlings grew.

### *Influence on enzyme activity of tobacco seedling*

The influence of different levels of nitrogen application on resistance-related POD, PPO, and PAL enzyme activities of tobacco leaves is shown in Table 1. The amount of nitrogen application significantly influenced the PAL activity of tobacco seedlings. PAL activity gradually increased as the seedlings grew. The PAL activities of the four processes at 51 days were lower than those at 45 days. This increase may be due to water filling in the nutrient solution, resulting in dilution of the solution. During later determinations (63 days),

PAL activities of T<sub>1</sub> and T<sub>4</sub> tended to decrease, which may have been caused by very low or excessive nitrogen content of late nutrient solution; the PAL activities of T<sub>1</sub>, T<sub>2</sub>, and T<sub>3</sub> were not significantly different.

Application of different amounts of nitrogen had a significant effect on the PPO activity of tobacco leaves (see Table 1). At 39 days, the PPO activity of T<sub>3</sub> was the highest while that of T<sub>2</sub> was the lowest; after 63 days, T<sub>2</sub> showed the highest PPO activity (644.55  $\text{U g}^{-1} \text{min}^{-1} \text{FW}$ ), which was 1.71 times that of T<sub>3</sub> (376.56  $\text{U g}^{-1} \text{min}^{-1} \text{FW}$ ). As the seedlings grew, the PPO activities of all processes increased (except for T<sub>1</sub>), of which T<sub>3</sub> had the highest increase in PPO activity, with 3.18-fold change from 39 days to 63 days.

Table 1 shows that POD activity decreased with increasing amount of nitrogen applied. At the beginning (39 days), T<sub>1</sub> had the lowest POD activity (28.34  $\text{U g}^{-1} \text{min}^{-1} \text{FW}$ ). Moreover, we found a slight difference in POD activity between the other three processes. The growth rate of POD activities in T<sub>1</sub> and T<sub>2</sub> increased gradually with the growth of tobacco seedlings, whereas the growth rate of POD activities in T<sub>3</sub> and T<sub>4</sub> were lower. In the late period (63 days after seeding), the POD activity in T<sub>2</sub> was the highest (i.e., 585.43  $\text{U g}^{-1} \text{min}^{-1} \text{FW}$ )—more than twice that of T<sub>4</sub>.

### *Influence on photosynthetic performance*

The influence of application of different amounts of nitrogen on the photosynthetic performance of tobacco seedlings is shown in Table 2. The amount of nitrogen applied had significant influence on  $P_n$  of tobacco seedlings.  $P_n$  gradually increased with increasing amounts of nitrogen application; T<sub>3</sub> showed a 17.29% increase compared with T<sub>1</sub>. The application of excessive amounts of nitrogen caused  $P_n$  to decrease.  $G_s$  was also significantly affected by the amount of nitrogen applied. If the amount of nitrogen applied was high,  $G_s$  increased. No significant differences were found between T<sub>1</sub> and T<sub>2</sub> or between T<sub>3</sub> and T<sub>4</sub>.  $C_i$  in T<sub>3</sub> was significantly higher than those in the three other processes, and no significant differences were found among T<sub>1</sub>, T<sub>2</sub>, and T<sub>4</sub>. The amount of nitrogen applied had a significant influence on  $T_r$ . Higher amounts of nitrogen applied tended to gradually increase  $T_r$  levels. After the amount of nitrogen applied reached a certain peak value,  $T_r$  decreased. Application of too low or excessive amounts of nitrogen decreased the  $WUE$  of tobacco seedlings.  $WUE$  of T<sub>2</sub> was the highest, and while significant differences were observed between T<sub>2</sub> vs. T<sub>3</sub> and T<sub>1</sub> vs. T<sub>4</sub>, no significant difference was observed between T<sub>2</sub> and T<sub>3</sub>.

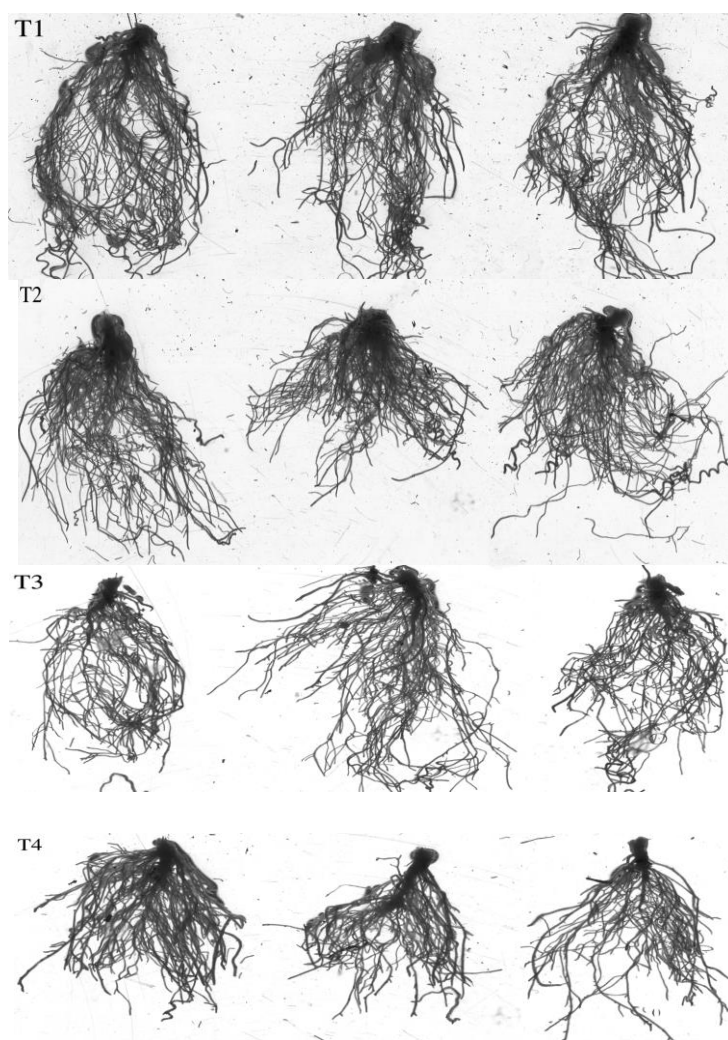
## Discussions

Tobacco roots have an important function not only in absorbing water and mineral nutrients but also in synthesizing nicotine and other active substances. Root growth has a direct effect on the yield and quality of tobacco leaves. The morphological properties of roots directly reflect their level of growth, development, and vitality. Here we discovered that low amounts of nitrogen application could stimulate tobacco seedlings to grow more fine and long roots. In contrast, high amounts of nitrogen application could promote the growth of short and thick roots. This growth may be related to fertilizer-induced growth of roots in a low-nitrogen environment, causing roots to continue to elongate to obtain more nutrients (Bonifas and Kimberly, 2009). The absorption capacity of fine roots is generally thought to be stronger than that of thick roots (Sullivan et al., 2000). Therefore, application of low amounts of nitrogen can enhance the absorption capacity of tobacco seedling roots.

**Table 1.** Effects of nitrogen treatments on POD, PPO, and PAL of tobacco seedling.

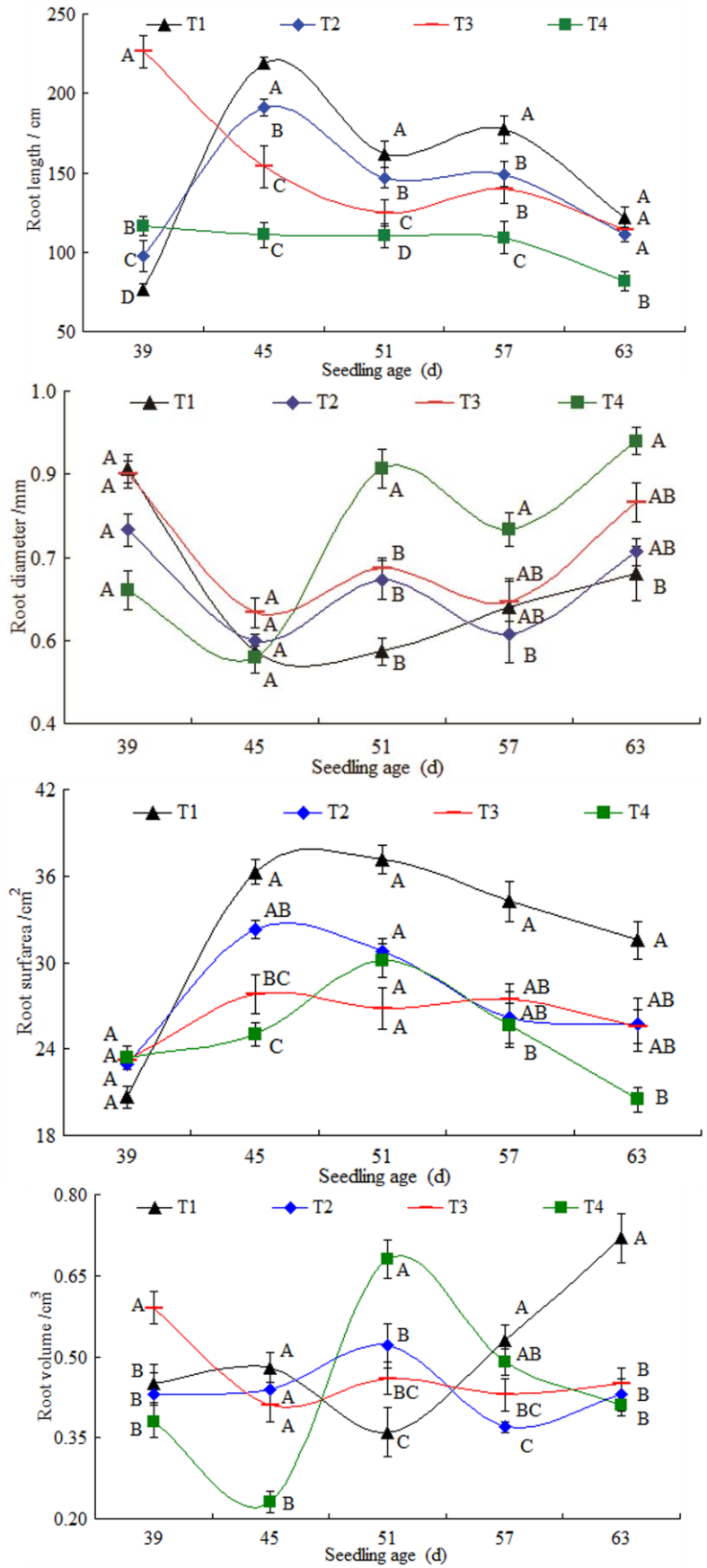
Item		<sup>§</sup> 39 d	45 d	51 d	57 d	63 d
PAL / U g <sup>-1</sup> min <sup>-1</sup> FW	T <sub>1</sub>	222.22 A	355.31 D	343.99 D	727.60 D	643.79 B
	T <sub>2</sub>	215.69 A	258.16 A	269.63 C	595.45 C	648.06 C
	T <sub>3</sub>	309.62 C	321.38 B	247.88 B	436.70 A	677.22 D
	T <sub>4</sub>	239.22 B	331.92 C	202.38 A	485.20 B	396.25 A
PPO /U g <sup>-1</sup> min <sup>-1</sup> FW	T <sub>1</sub>	242.92 C	361.46 D	447.40 D	447.40 D	407.09 B
	T <sub>2</sub>	202.61 A	296.61 C	412.18 C	412.18 C	644.55 C
	T <sub>3</sub>	251.10 D	282.40 B	380.70 B	380.70 B	384.17 A
	T <sub>4</sub>	220.60 B	220.32 A	233.07 A	233.07 A	376.56 A
POD / U g <sup>-1</sup> min <sup>-1</sup> FW	T <sub>1</sub>	28.34 A	146.17 A	192.04 C	403.07 D	556.74 B
	T <sub>2</sub>	123.27 B	144.59 A	146.31 B	285.88 C	585.43 B
	T <sub>3</sub>	146.48 C	184.68 B	156.00 B	188.03 B	364.85 A
	T <sub>4</sub>	127.98 B	145.39 A	124.49 A	152.17 A	290.67 A

Note: <sup>§</sup> the days mean after seeding. Different capital letters stand for significant differences at 0.01 levels.

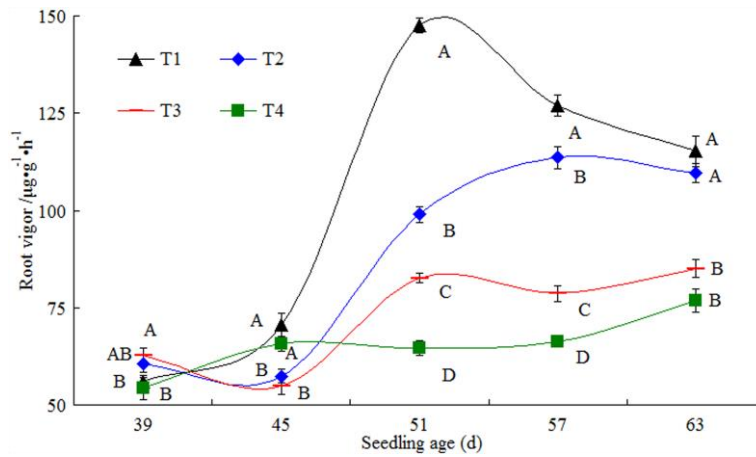
**Fig 1.** The scanning photos of tobacco seedling's root morphological characteristics**Table 2.** Effects of nitrogen treatments on photosynthetic characteristics of tobacco seedling

Treatment	<sup>†</sup> <i>P<sub>n</sub></i>	<i>G<sub>s</sub></i>	<i>C<sub>i</sub></i>	<i>T<sub>r</sub></i>	<i>WUE</i>
T <sub>1</sub>	20.01 a	0.16 a	586.50 a	1.26 a	15.88 a
T <sub>2</sub>	22.47 c	0.16 a	594.46 a	1.33 b	16.89 b
T <sub>3</sub>	23.47 d	0.17 b	618.75 b	1.44 d	16.30 b
T <sub>4</sub>	21.69 b	0.17 b	588.30 a	1.40 c	15.49 a

Note: <sup>†</sup>*P<sub>n</sub>*, net photosynthetic rate; *G<sub>s</sub>*, stomatal conductance; *C<sub>i</sub>*, intercellular CO<sub>2</sub> concentration; *T<sub>r</sub>*, transpiration rate; *WUE*, water use efficiency. Different litter letters stand for significant differences at 0.05 levels.

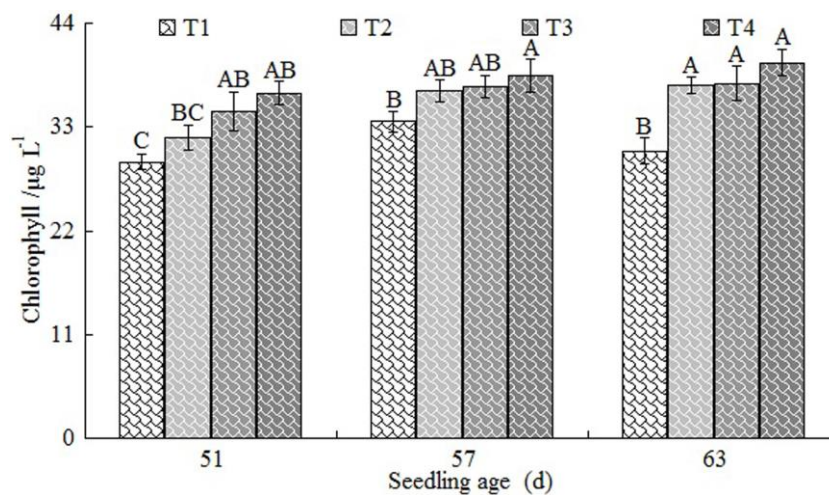


**Fig 2.** Effects of nitrogen treatments on morphological features of tobacco seedling roots.  
 Note: Mean values in same seedling age with different capital letters are significantly differences using Duncan tests at 1% of probability.



**Fig 3.** Effects of nitrogen treatments on root vigor of tobacco seedling.

Note: Mean values in same seedling age with different capital letters are significantly differences using Duncan tests at 1% of probability.



**Fig 4.** Effects of nitrogen treatments on the Chlorophyll of tobacco seedling leaf.

Note: Mean values in same seedling age with different capital letters are significantly differences using Duncan tests at 1% of probability.

Jiang et al. (2008) has also reported similar findings. Root activity reflects the intensity of metabolic capacity of the roots and directly affects the growth and resistance of tobacco seedlings. Furthermore, it also has a crucial effect on the revival period after the seedling is transplanted to large open fields. Several reports have shown that the root activity of plants increases with the application of increasing amount of nitrogen, but starts to decrease beyond a certain level of nitrogen (Jiang et al., 2008; Cao, et al., 2012). Our findings are consistent with these results. At the beginning, no significant difference in root activity was found between plants that received different amounts of nitrogen. In the middle and late phases of evaluation, the root activity of tobacco seedlings decreased with increasing amount of fertilizer applied.

Chlorophyll, an important pigment for plant photosynthesis, can capture light energy and drive electrons to the reaction center (Fromme et al., 2003). It has a crucial effect on the growth and development of plants, which ultimately affects crop yield and quality. Nitrogen is a component of chlorophyll molecules. Its shortage can affect chlorophyll formation. Xie et al. (2007) showed that the amount of nitrogen applied has a strong effect on the chlorophyll content of flue-cured tobacco: chlorophyll content shows an increasing trend with increasing amounts of applied nitrogen.

Other scholars found that the chlorophyll contents of winter wheat (Yang et al., 2008), spring maize (Liu et al., 2011), cotton (Wu et al., 1999), and other crops have similar responses to nitrogen. The current study shows that the chlorophyll content of tobacco gradually increased with increasing amounts of nitrogen applied. Tobacco seedlings are susceptible to diseases in floating-bed conditions because of high density and high humidity, in addition to several other reasons. Applying excessive amounts of nitrogen causes high chlorophyll contents and leaf hypertrophy, further aggravating crown closure of seedling trays and increasing the risk of disease in tobacco seedlings.

POD, PPO, and PAL activities in tobacco are closely related to disease resistance (Chen et al., 1994; Liu et al., 2003; Pan et al., 2004). When pathogens infect tobacco seedlings, these enzymes are rapidly activated to prevent the pathogen from progressing further. This study shows that the level of nitrogen application had significant influence on POD, PPO, and PAL activities in tobacco seedlings. In the middle phase of evaluation, increasing the amount of nitrogen applied decreased the activity of these three enzymes. T<sub>1</sub>, which had received the lowest amount of nitrogen, showed the highest activity (except for POD at 39 days). In later evaluations, T<sub>2</sub> showed the highest PPO and POD activities, whereas T<sub>3</sub> showed the highest PAL activity.

This result indicates that excessively high or excessively low nitrogen levels decreased the activity of the three enzymes, thus reducing the resistance of tobacco seedlings. Liu et al. (2012) also observed similar results in cotton. Some studies have also shown that the enzyme activity of POD in tobacco leaves increases slightly with increasing amounts of applied nitrogen (Fu et al., 2009).

$P_n$  reflects the photosynthetic capacity of a plant to a certain extent. The nutrient level of nitrogen has a significant regulatory effect on  $P_n$  (Zhao, 2006). This study shows that the amount of nitrogen applied had a significant influence on  $P_n$  in tobacco seedlings. As the amount of nitrogen applied increased,  $P_n$  also increased. However, excessive amounts of nitrogen fertilizer caused  $P_n$  levels to decrease.  $C_i$ ,  $T_r$ , and WUE initially increased and then decreased with increasing amounts of applied nitrogen. Yun et al. (2010) found that  $P_n$ ,  $C_i$ ,  $T_r$ , and WUE in flue-cured tobacco leaves are the highest in the middle phase of nitrogen application. However, Fu et al. (Fu et al., 2012) found that  $P_n$  and  $T_r$  of tobacco increased significantly during the large field stage of planting with increasing amounts of applied nitrogen. Feng et al. (2008) found that  $P_n$  initially increased and then decreased with increasing amount of applied nitrogen in Fuji apples. Some studies have shown that the WUE of rice leaves also increased with increasing amount of nitrogen applied (Xiao et al., 2013). Considering the poor resistance of tobacco seedlings and the environment it is grown in, which make it susceptible to diseases, application of excessive amounts of nitrogen can cause accumulation of photosynthesis products, leaf hypertrophy, and seedling tray crown closure, further aggravating the risk of disease outbreaks. Therefore, when growing tobacco seedlings in floating beds, the amount of nitrogen applied must be controlled to avoid hypertrophy of young seedlings and leaves, which increases the risk of disease.

## Materials and Methods

### Plant materials

The plant material was tobacco (*Nicotiana tabacum* L.) cultivar K326.

### Design of experiments

Experiments were performed in a greenhouse at Southwestern University using K326 flue-cured tobacco. This tobacco variety was seeded on February 22, 2014 using floating bed technology. A total of four nitrogen treatment conditions were set up, namely,  $T_1$  (6 mg/plant),  $T_2$  (11 mg/plant),  $T_3$  (16 mg/plant), and  $T_4$  (21 mg/plant). Total nitrogen applied to each treatment group was calculated based on pure nitrogen content. The four seeding trays in each treatment group were placed in stainless steel water trays and processing was done three times. The amounts of phosphate fertilizer and potassic fertilizer applied were 11 mg/plant and 29 mg/plant, respectively. All fertilizers were applied once in the full stand stage. The trays were irrigated once every 3 to 4 days to maintain a nutrient level depth of  $4 \pm 1$  cm.

### Sample collection and index determination

#### Root scanning

Samples of tobacco seedling roots were collected at 39, 45, 51, 57, and 63 days after seeding. The roots were washed

with clean water, processed (30 plants/tray), and scanned using the Epson Root Scanner (Japan). Images of the root system were captured, and root length, average root diameter, root surface area, root volume were analyzed with the software WINRHIZO.

#### Determination of root activity

Samples of tobacco seedling roots were collected at 39, 45, 51, 57, and 63 days after seeding (30 plants/tray) and processed to determine root activity using the TTC reduction method. This method has been arelady described in detail in the literature (Zhang and Qu, 1990).

#### Determination of enzyme activity

Samples of tobacco leaves were collected at 39, 45, 51, 57, and 63 days after seeding (30 plants/tray) and processed to determine relative enzyme activity. The activity of phenylalanine (PAL) ammonia-lyase was determined using a spectrophotometer (Wang et al., 1981). The activities of polyphenol oxidase (PPO) were determined by the catechol method (Zhu et al., 1991) and the hydrogen peroxide oxidation method (Zhou, 2000).

#### Determination of chlorophyll

Samples of tobacco seedling leaves were collected at 51, 57, and 63 days after seeding to determine the chlorophyll content using a spectrophotometer colorimetric method after acetone extraction (Zhou, 2000).

#### Determination of photosynthetic performance

Photosynthetic performance was determined from 10:00 am to 11:00 am, 58 days after seeding. Before determination, tobacco seedlings were removed from the greenhouse to allow them to receive sunshine for 2 h or more. The Li-6400 Portable Photosynthesis System manufactured by Licor (USA) was used to determine the photosynthetic rate of tobacco seedling leaves ( $P_n$ ), intercellular  $CO_2$  content ( $C_i$ ), transpiration rate ( $T_r$ ), stomatal conductance ( $G_s$ ) and water use efficiency, which was calculated as  $P_n/T_r$ . System control conditions were set according to the following standards:  $CO_2$  content, 360  $\mu\text{mol/mol}$ ; light intensity, 1200  $\mu\text{mol/m}^2\cdot\text{s}$ ; and temperature, 25 °C.

## Conclusion

The amount of nitrogen applied during the growth of tobacco seedlings in floating beds had significant influence on the morphological properties of roots, root activities, leaf photosynthetic performance, and plant resistance. This study shows that low nitrogen level facilitated increase in the number of fine roots, higher root activity, and enhanced root absorption capacity in tobacco seedlings. It also increased the activities of POD, PPO, and PAL, which are related to the disease-resistance capacity of tobacco seedlings. The amount of nitrogen applied also had significant influence on the chlorophyll content and photosynthetic performance of tobacco seedlings. Furthermore, significant enhancement in  $P_n$ ,  $C_i$ ,  $T_r$ , and WUE were observed with nitrogen supplementation. Thus, after a comprehensive analysis of the influence of nitrogen application on the morphological properties and activities of roots, as well as the physiological and photosynthetic performance of the leaves, we propose that applying nitrogen at 6 to 11 mg/plant to tobacco



seedlings grown by the floating-bed method facilitates growth of healthy tobacco seedlings with strong roots and good resistance.

### Acknowledgements

The authors thank the Chongqing Tobacco Monopoly Bureau (Item Number: NY20150601070011) for funding this work. The authors also thank Li Najia, Li Huachuan for their assistance in sample testing.

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