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ORIGINAL ARTICLE

The phenotype and pathogenicity of *Ralstonia solanacearum* transformed under prolonged stress of excessive exogenous nitrogen

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Abstract

Bacterial wilt, caused by soil-borne pathogen *Ralstonia solanacearum*, is a serious disease in many plants such as Solanaceae. To investigate the effects of accumulated nitrogen in soil on the phenotype and pathogenicity of the *R. solanacearum*, a serial passage experiment (SPE) was designed. Specifically speaking, minimal medium supplied with a slight excess of ammonium sulphate (AS) or ammonium nitrate (AN) was used to simulate the nutrition of soil containing excess nitrogen. During the period of 30 SPE, the phenotype, pathogenicity and relative expression of nitrogen metabolism genes in *R. solanacearum* were monitored. Phenotypic analysis results illustrated that the colony morphology of *R. solanacearum* changed after long-term culture, from high virulence colonies with strong fluidity to small, round non-mucoid colonies; The strain after prolonged stress of excessive exogenous nitrogen was a no-virulence phenotype conversion type (PC-type). The time for a change in colony morphology to occur after exposure to exogenous AS or AN was significantly less than the untreated samples, which treated without exogenous nitrogen. The results of pathogenicity also demonstrated that the cultures treated with exogenous AN or AS reduced virulence more quickly than the control. The disease index of 10 SPE with AN treatment or AS treatment was 89% or 68% lower than that of the control, respectively. In addition, as the incubation time increased, the swimming motility and the number of biofilms formation of the cultures were significantly changed under both treatments in comparison to the untreated samples. Furthermore, the relative expression of the nitric oxide reductase *norB* gene in the cultures treated with AN was 1.51-fold higher compared with the control after 30 SPE. These results indicated that excessive nitrogen supply in the environment could accelerate the transformation of *R. solanacearum* from high virulence wild-type into a PC-type, probably for the purpose of adapting to the adverse environment.

KEYWORDS

excessive nitrogen, nitrogen metabolism gene, phenotype conversion, *Ralstonia solanacearum*, serial passage experiment

1 | INTRODUCTION

Bacterial wilt is an important disease caused by *Ralstonia solanacearum*, which has a wide range of hosts and causes huge economic losses (Hayward, 1991). Recently, the scope of damage from the disease has increased due to the climate, soil conditions, farming systems and other changes (Jiang et al., 2017). *Ralstonia solanacearum* invades plants through wounds in the root or stem of the host, then enters the xylem and spreads to the upper part of the plant, producing a large amount of extracellular polysaccharide (EPS) that causes vascular obstruction (Chen et al., 2018). At the same time, EPS is also secreted to degrade the cell wall of the host plant, which leads to the rapid wilting of the host plant. Without a host plants, *R. solanacearum* can survive in the soil for a long time. Therefore, the soil environment is an important living place for *R. solanacearum*.

The overuse of chemical fertilizer is now a serious issue. Nitrogen fertilizer is one of the most important fertilizers in agriculture. However, the excessive application of nitrogen fertilizer leads to the accumulation of inorganic nitrogen and changes in physical and chemical properties. Recent research about nitrogen on plant pathogens mainly focused on the effect of nitrogen application on soil microbial community structure in the field (Wang, Xiao, et al., 2018). The concentration of water-soluble nitrogen in soil correlated strongly with the aggressive invasion of soil pathogens (Wei et al., 2018). A previous report showed that when 1 mol of ammonium ion was absorbed in soil, two moles of hydrogen ions were released (Wang, Zhang, Zhao, & Xu, 2018; Wang, Xiao, et al., 2018). With the continuous transformation of nitrogen fertilizer in the soil environment, the physiological activities of releasing hydrogen ions will continuously decrease the pH of soil (Lauber, Hamady, Knight, & Fierer, 2009; Zhalnina et al., 2015). Soil acidification is a very serious problem worldwide (Alkan, Espeso, & Prusky, 2013). The excessive application of nitrogen fertilizer plays a certain role in the progress of soil acidification (Guo et al., 2010).

Studies have reported that soil acidification has a positive effect on *R. solanacearum* and on the outbreak of bacterial wilt. On the one hand, acidic soil conditions (pH 4.5–5.5) are beneficial to the growth of *R. solanacearum* and inhibit anti-microorganisms of *R. solanacearum* such as *Pseudomonas fluorescens* and *Bacillus cereus* (Alkan et al., 2013). On the other hand, the acidic environment is beneficial to the expression of virulence genes in *R. solanacearum* and weakens the defence response of host against *R. solanacearum* (Garbeva, van Veen, & van Elsas, 2004; Manteau, Abouna, Lambert, & Legendre, 2003; Ownley, Weller, & Thomashow, 1992; Rousk et al., 2010).

To a certain extent, there is a certain correlation among soil acidification, nitrogen fertilizer and *R. solanacearum*. Previous research has studied the relationship between soil acidification and *R. solanacearum* (Li et al., 2017). However, whether the accumulation of nitrogen in soil influences *R. solanacearum* is still unknown. Nitrogen is necessary not only for the growth and development of plants but also for *R. solanacearum* growth. In this study, minimal medium was selected to simulate the barren state of the soil, and ammonium sulphate (AS) or ammonium nitrate (AN) was added to simulate the accumulation of inorganic nitrogen. Under this condition, *R. solanacearum* was cultured for a long time. During the culture progress, the characteristics of *R. solanacearum* were monitored to explore the possible adaptation of *R. solanacearum* under long-term interaction with a slight excess of nitrogen.

This study simulated the direct effect of nitrogen accumulation on *R. solanacearum* under similar soil conditions and discussed the changes in phenotype and pathogenicity during this process.

2 | MATERIALS AND METHODS

2.1 | Serial passage experiment

A serial passage experiment (SPE) was performed on a clone of *R. solanacearum* CQPS-1 (Li et al., 2017) using minimal medium (MM medium) supplemented with 0.05 mol/L AS ($(NH_4)_2SO_4$) or AN (NH_4NO_3), with normal MM medium as the control. The composition of MM is as follows (Boucher, Barberis, Trigalet, & Demery, 1985): $FeSO_4 \cdot 7H_2O$, 1.25×10^{-4} g/L; $(NH_4)_2SO_4$, 0.5 g/L; $MgSO_4$, 0.03 g/L; KH_2PO_4, 3.4 g/L, Glucose, 0.99 g/L. The pH of MM was adjusted to 6.5 with KOH. At each SPE, bacteria were grown in 20 ml medium for 24 hr at 30℃ with 180 r/min shaking, and then 100 μl of culture was transferred into 20 ml medium; each treatment had five independent cultures. The $OD_{600\ nm}$ value of the culture was measured every day. When medium containing bacteria was transferred to a new medium, it was called one SPE. Glycerol stock of strains stored at −80℃ at point time. When evaluated the phenotype and pathogenicity, the strains (including five, 10, 15 and 30 SPE) from their −80℃ glycerol stock were cultured on BG medium (Boucher et al., 1985).

2.2 | Biofilm formation

The culture with a significant change in $OD_{600\ nm}$ in the SPE process was selected, which were 5, 10, 15 and 30 SPE cultures. The biofilm formation of *R. solanacearum* was detected by 96-well polystyrene microtiter plates according to Yang et al. (2016).

2.3 | Swimming motility

The swimming motility of 5, 10, 15 and 30 SPE cultures was determined according to Perrier et al. (2016). Briefly, bacteria were grown in MM medium supplemented with 20 mM glucose for 1 day at 30°C. The bacterial cultures were grown to the logarithmic stage and diluted to $OD_{600\ nm}$ = 0.1 (approximately 1.0×10^8 cfu/ml) with aseptic water. Then, 2.5 μl of culture was stab-inoculated into the centre of MM medium semi-solid agar (0.3%) plates supplemented with 20 mM glucose. Five plates were inoculated for each culture, and the experiment was repeated three times. The culture dish was placed in a 30℃ constant temperature incubator. After 72 hr of culture, swimming motility of the cultures was recorded.

2.4 | Pathogenicity

The pathogenicity of the selected cultures was tested on 8-week-old tobacco plants (cv. Yunyan 87, a susceptible cultivar; YuxiZhong Yan Tobacco Seed CO., LTD). The overnight bacterial cultures in B medium were diluted with aseptic water to 10^6 cfu/ml. The bacteria were inoculated via stem injection (Morel et al., 2018). Briefly, 10 μl of bacterial suspension was injected into the stem of the plant, 0.5 cm above the root, using a microsyringe. This experiment was repeated 3 times with 5 plants for each culture per replicate. The incidence and disease index were monitored every day for 14 days using a 0–4 scale as previous described (Lebeau et al., 2013).

2.5 | Relative expression of nitrogen metabolism genes

The relative expression of the genes encoding the complete denitrification pathway in the genome of 30 SPE cultures was detected, including *narG* (nitrate respiratory reductase), *aniA* (nitrite reductase), *norB* (nitric oxide reductase) and *nosZ* (nitrous oxide reductase). The selected cultures were cultured to $OD_{600\ nm}$ = 0.5 in MM medium supplemented with a concentration of 0.05 mol/L N, centrifuged for 10 min to remove medium and then frozen in liquid nitrogen. RNA extraction and quantitative real-time PCR (qRT-PCR) were performed according to a previous report (Yang et al., 2018). The primers of each gene used for qRT-PCR were designed by Primer3 page (http://primer3.ut.ee/) and the specificity was verified (Table 1). The housekeeping gene *serC* was used as the internal reference gene (Wu, Ding, Zhang, Liu, & Yang, 2015).

2.6 | Data analysis

The software of SPSS (version 17.0) was used for statistical analysis. The difference among multiple treatments was compared and statistically analysed through one-way analysis of variance with Tukey method, and the ORIGIN software (version 9) was used to draw the figure.

3 | RESULTS

3.1 | Correlation between the changes in OD values and colony morphologies of the cultures

Bacteria were cultured under long-term stress of exogenous AS and AN with daily passage for 30 days. The $OD_{600\ nm}$ of the cultures were monitored every day. According to the results, the $OD_{600\ nm}$ of the cultures changed greatly in the early stage of the long-term culture and tended to be stable at the later stage (Figure 1). Initially, the amount of cultures treated by AN was superior to the nitrogen treatment and the control. During the culture growth period, there was a point during each treatment when the cultures barely grew (the $OD_{600\ nm}$ was close to 0, Figure 1). The time for the AN treatment to reach the point of minimal growth was the earliest (after five times of SPE), the AS treatment was second (after 10 times of SPE), and the control was last (after 15 times of SPE). However, the cultures continued to grow when uninterruptedly transferred into new, fresh medium. The colony morphology of the cultures grown to this point was observed (Figure 2a). The results showed that the colony morphology changed significantly from high virulence colonies with strong fluidity (Figure 2b) to small round non-mucoid colonies (Figure 2b). According to this phenomenon, which were called 5, 10, 15 and 30 SPE, respectively, were selected to evaluate the phenotype and pathogenicity.

3.2 | Effect of nitrogen treatments on biofilm formation during long-term culture

Biofilm formation was carried out on the cultures 5, 10, 15 and 30 SPE. Comparing the amount of biofilm at each point, the 5, 10 and 15 cultures showed no significant difference in biofilm formation within the three treatments (figures not shown), but there were significant differences between the AS treatment and the control at 30 SPE. The average $OD_{530\ nm}$ of the AS treatment was 0.59, the average value of the control was 0.23, and the number of biofilms treated with AS was approximately 2.5 times that of the control (Figure 3a). Considering the effect of the SPE times on the amount of biofilm, the control exhibited no difference within the different SPEs (Figure 3b). However, the amount of biofilm increased gradually among the different SPE times treated by AS and AN (Figure 3c,d).

3.3 | Effects of nitrogen treatments on swimming motility during long-term culture

We measured the swimming motility of the 5, 10, 15 and 30 cultures. The comparison of swimming halo diameters of different cultures at the same time point showed that the swimming halo diameter of the cultures treated with AN in five SPE was 0.83 ± 0.25 mm, and the control was 0.5 ± 0.13 mm (Figure 4a). The swimming halo diameter of the cultures treated with AS in 10 SPE was 3.31 ± 0.45 mm, and the control was 1.93 ± 0.74 mm (Figure 4b). However, there were no significant differences between the three treatments at 15 and 30 SPE (Figure 4c,d).

3.4 | Effects of nitrogen treatments on pathogenicity during long-term culture

The pathogenicity of the 5, 10 and 30 SPE cultures was evaluated by stem injection under greenhouse conditions. The results of the disease index showed that the virulence of the five SPE culture was reduced by 24% after AN treatment compared to AN treatment of the control at the same time point (Figure 5a). When comparing the virulence of the 10 SPE cultures from different treatments, the

TABLE 1 Sequences of the primers used for qRT-PCR in this study

Gene	Upstream primer (F) (5′–3′)	Downstream primer (R) (5′–3′)
NarG	GCAGTTCTACCAGGACCACC	ATCCAGTTCAGCACCAGCTC
AniA	GGAGGTGGTCGAGAAGGAAA	AAGGTGAACTGCGATTCGTG
NorB	GGCATGCAGCGCTGTTC	GCAGCGACATGAACACCATC
NosZ	CACCCTGATCCTCACCAACC	GGCAGAAATGCGTGCAGTAG
SerC	CCCACCTACGCCATCTATGT	TTGAGGAAGAACGGCACATT

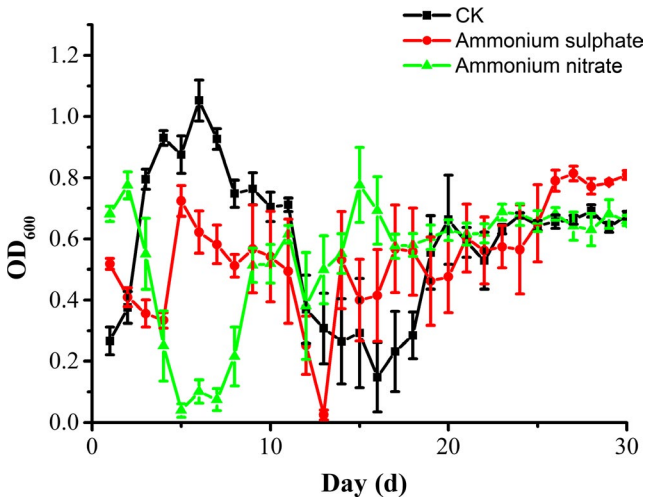

FIGURE 1 Optical density (OD) values of different treatments within 30 days [Colour figure can be viewed at wileyonlinelibrary.com]

results showed that the virulence of the 10 SPE cultures from two nitrogen treatments was significantly reduced; the AN treatment was 89% lower than the control and that of the AS treatment was 68% lower than the control (Figure 5b). Interestingly, when the 30 SPE cultures were tested, the results showed that there were no differences among the three treatments, and all of the cultures from the three treatments did not cause bacterial wilt (figure not shown).

3.5 | Relative expression of nitrogen-metabolizing enzyme genes after long-term culture

The relative expression levels of nitrogen-metabolizing enzyme genes, including *narG*, *aniA*, *norB* and *nosZ* (Dalsing & Allen, 2014), were determined by qRT-PCR. The results showed that the relative expression of *norB* in the cultures after long-term treatment by AN was 1.51-fold higher compared with that of the control. (Figure 6c). There was no significant difference in the relative expression of *narG*, *aniA*, or *nosZ* between the three treatments (Figure 6a,b,d).

4 | DISCUSSION

According to the results of this study, AS and AN promoted the growth of *R. solanacearum* for a short time at the beginning of SPE. However, after a period of time (approximately four times of SPE), the growth of the cultures in the AS and AN treatments was lower than that in the control (Figure 1). At the end of SPE, the OD values of the cultures in AS were significantly higher than those in AN or in the control. In general, the growth of the cultures in all treatments at the end stage of the SPEs tended to be stable, which might be the result of cultures adapting to the growth environment.

It was demonstrated that *R. solanacearum* spontaneously loses virulence ability under the condition of poor soil or artificial culture medium. This progress was called PC-type (Poussier et al., 2003). The PC-type of *R. solanacearum* was a form that specifically adapted for survival in poor environments, such as the soil, plant debris or starving conditions (Denny, Brumbley, Carney, Clough, & Schell, 1994). PC-type cultures stop secreting virulence factors such as extracellular polysaccharides. At the same time, the swimming motility is enhanced, and the infection ability is improved. In this study, morphological observations were carried out on the time point at which the OD value of the culture changed significantly during SPE. Interestingly, before changing to the PC-type, the growth of the culture stopped (Figures 1 and 2). The OD value of the culture would approach 0 when the culture changed transitioned from the colony shape with strong fluidity to the colony form with weak mobility. Moreover, the time when *R. solanacearum* underwent PC-type in certain conditions was different. The time for cultures to transform was earliest when cultured in the AN treatment. The phenomenon in which the pathogenicity was significantly weakened and the swimming motility was significantly improved was also observed in this study. *Ralstonia solanacearum* grown in the AN treatment was the earliest to lose pathogenicity. In the detection of the five SPE cultures, the disease index of the culture treated with AN was lower than the control, and in the detection of the 10 SPE cultures, the two nitrogen treatments were lower than the control. This is also an important feature of the weakened pathogenicity of *R. solanacearum*.

Motility is one of the most important pathogenicity factors of *R. solanacearum* (Hayward, 1991). The motility of *R. solanacearum* is reflected in two aspects: swimming motility and twitching motility. Swimming motility is controlled only by flagellum, but flagellum formation and movement in *R. solanacearum* are regulated by very complex genes and are influenced by cell concentrations and other signalling molecules, such as nitrate (Dalsing & Allen, 2014; Ray, Kumar, Peeters, Boucher, & Genin, 2015; Tans-Kersten, Brown, & Allen, 2004). In general, changes

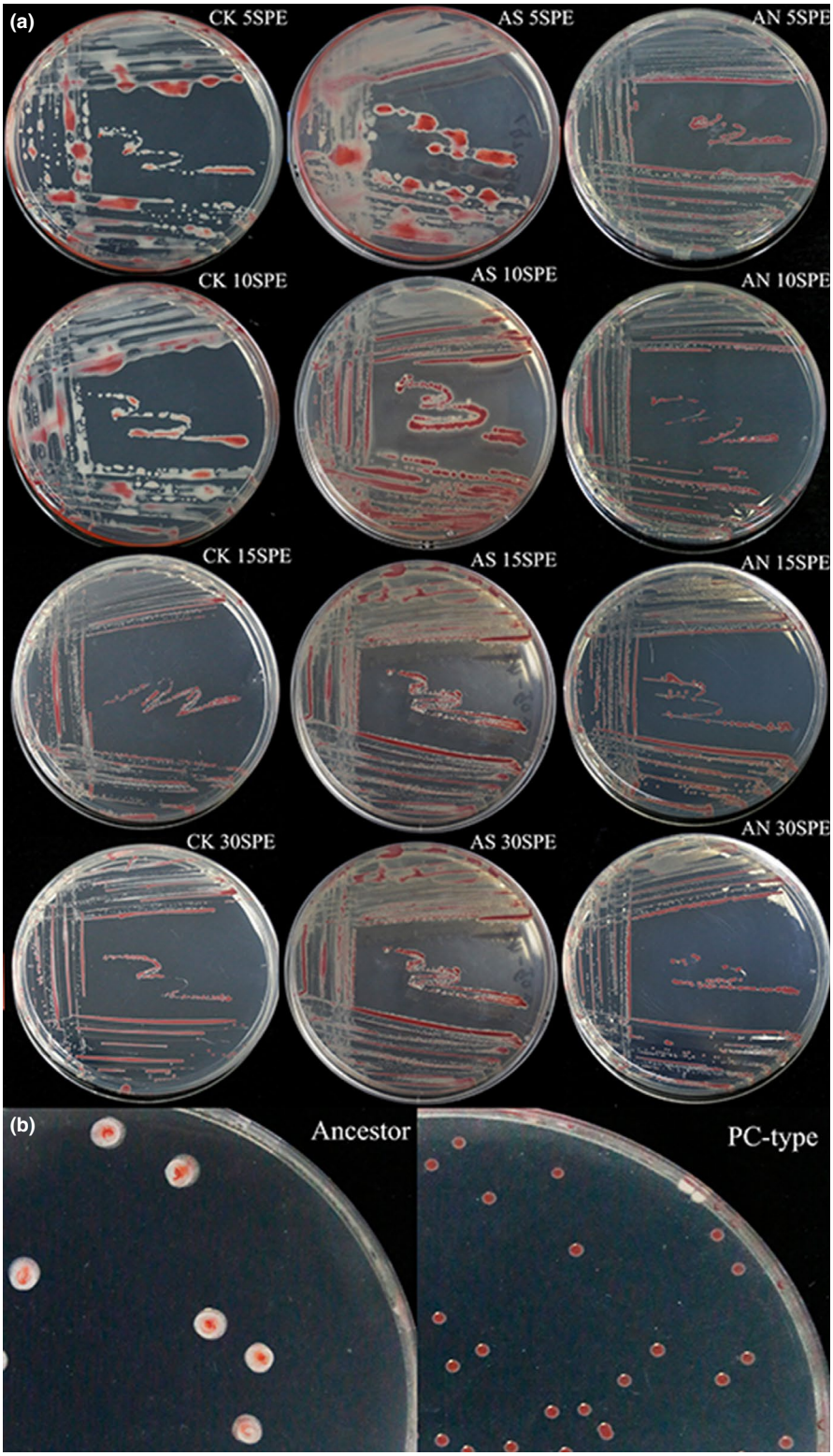

FIGURE 2 Colony morphology of *Ralstonia solanacearum*. (a) Colony morphology of different serial passage experiment (SPE) treated by ammonium sulphate (AS), ammonium nitrate (AN) and the control treated without exogenous nitrogen (CK) on B plates, (b) Morphology of individual colony of Ancestor and PC-type strain [Colour figure can be viewed at wileyonlinelibrary.com]

in mobility contribute to the adaptation of *R. solanacearum* to a variety of environments. To maintain its pathogenic mechanism and population continuation, *R. solanacearum* needs self-regulation to achieve the balance of pathogenicity, exercise, replication and metabolism (Jiang et al., 2017). Under the SPE in a slight excessive of exogenous nitrogen treatment, AN treatment first enhanced *R. solanacearum* swimming motility, and AS treatment then enhanced the swimming motility of the culture. It is inferred from the results that, in the process of adapting to lightly excessive exogenous nitrogen, the phenotypes of *R. solanacearum* that contribute to stress resistance, such as swimming motility and biofilm, are enhanced to a certain extent, while the pathogenicity is relatively weakened. This forms a relative balance of its physiological activities so that *R. solanacearum* has an enhanced ability to survive and reproduce.

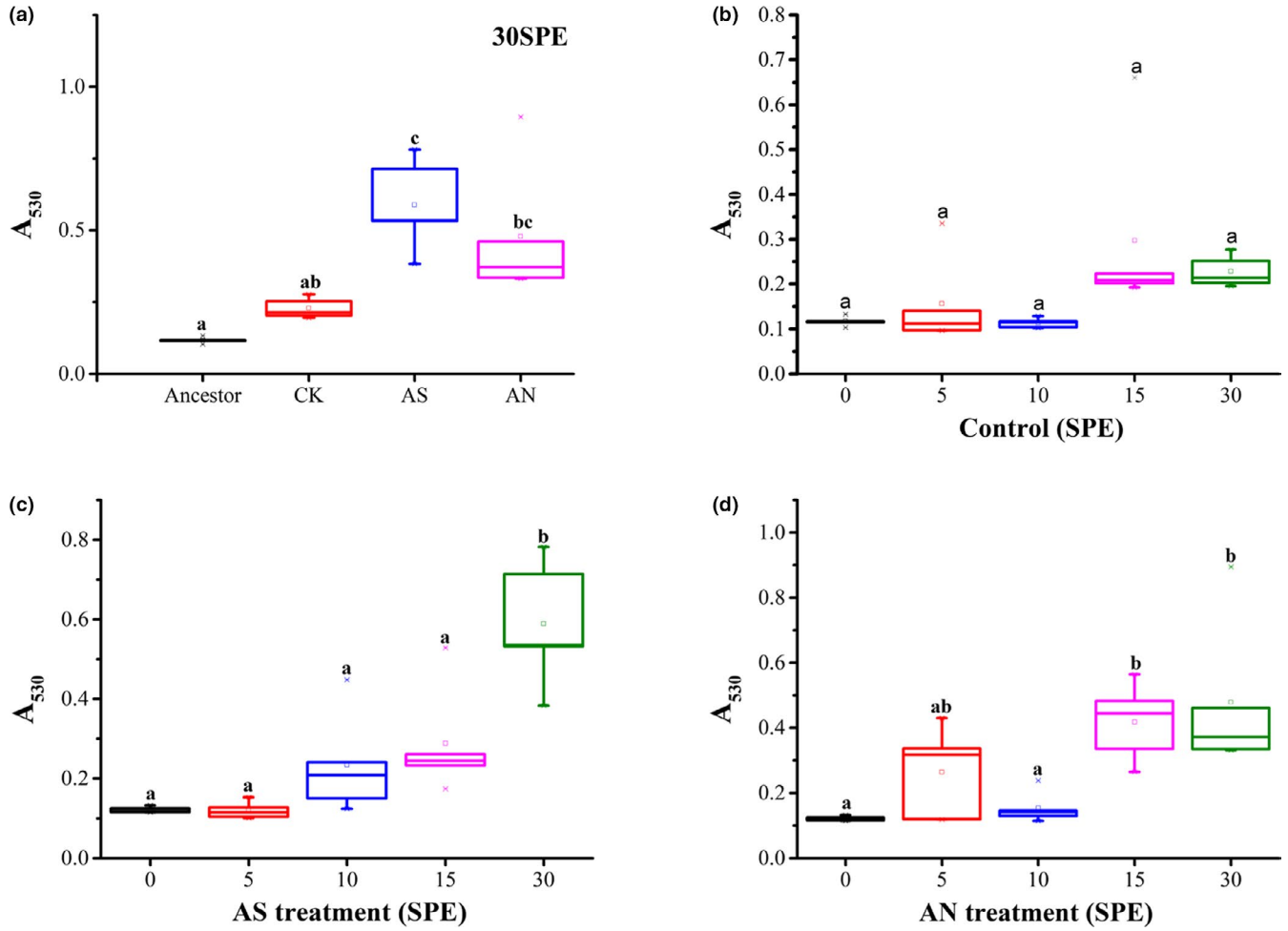

FIGURE 3 Effects of different treatments on the biofilm formation of *Ralstonia solanacearum*. (a) Difference of biofilm formation in 30 serial passage experiment (SPE) of each treatment. (b) Difference of biofilm formation between each SPE of the control treated without exogenous nitrogen (CK). (c) Difference of biofilm formation between each SPE of ammonium sulphate (AS). (d) Difference of biofilm formation between each SPE of ammonium nitrate (AN). Lowercase letters indicate a significant differences ($p < .05$) [Colour figure can be viewed at wileyonlinelibrary.com]

Biofilms can help bacteria resist harsh environments, such as soil drought and harmful substances (Danhorn & Fuqua, 2007; Morris & Monier, 2003). The results showed that the biofilm of the two nitrogen treatments fluctuated with increasing SPE time, but the control was mainly stable. This indicates that in the long-term culture process, nitrogen can have a certain effect on the resistance of the cultures to adverse environments, but the effect of nitrogen on biofilms is smaller than the effect of nitrogen on other phenotypes.

Dalsing and Allen (2014) showed that in the anoxic environment of tomato xylem, *R. solanacearum* can use nitrogen instead of oxygen as a terminal electron acceptor to complete the processes of growth metabolism and virulence infection. Nitrate can act the terminal electron receptor and help complete the respiration, growth and pathogenicity of *R. solanacearum*, while nitrite and nitric oxide do not play a direct role in the growth and pathogenicity of *R. solanacearum*. In this study, the response of the nitrogen-metabolizing enzyme gene of *R. solanacearum* was analysed from the level of gene expression. The results showed that after long-term subculture, the relative expression of the *norB* gene in *R. solanacearum* was increased by AN treatment. The results indicated that the culture could resist the action of NO toxic bacteria to some extent and enhance the survival ability of the culture.

From the results of this paper, we can see that the long-term presence of a slight excessive of exogenous nitrogen had an effect on the phenotype and pathogenicity of *R. solanacearum*. The base medium and tiny amount of nitrogen provide a harsh stress for *R. solanacearum*, which can rapidly transform into the PC-type. The biofilm and swimming motility of *R. solanacearum* also changed accordingly to cope with changes in the environment. Wang, Zhang, et al. (2018) and Wang, Xiao, et al. (2018) showed that nitrogen is one of the necessary factors to cause soil acidification. *Ralstonia solanacearum* can grow well (enhanced infection ability and pathogenicity) in an environment of pH = 6. This study revealed the effect of a slight excessive of exogenous nitrogen on *R. solanacearum*. Under the direct influence of nitrogen, the transformation of *R. solanacearum* to PC-type is accelerated. The PC-type has weak pathogenicity and strong mobility, which provides the cultures a stronger survival potential in poor environments. This type still maintains infection ability, which makes the cultures

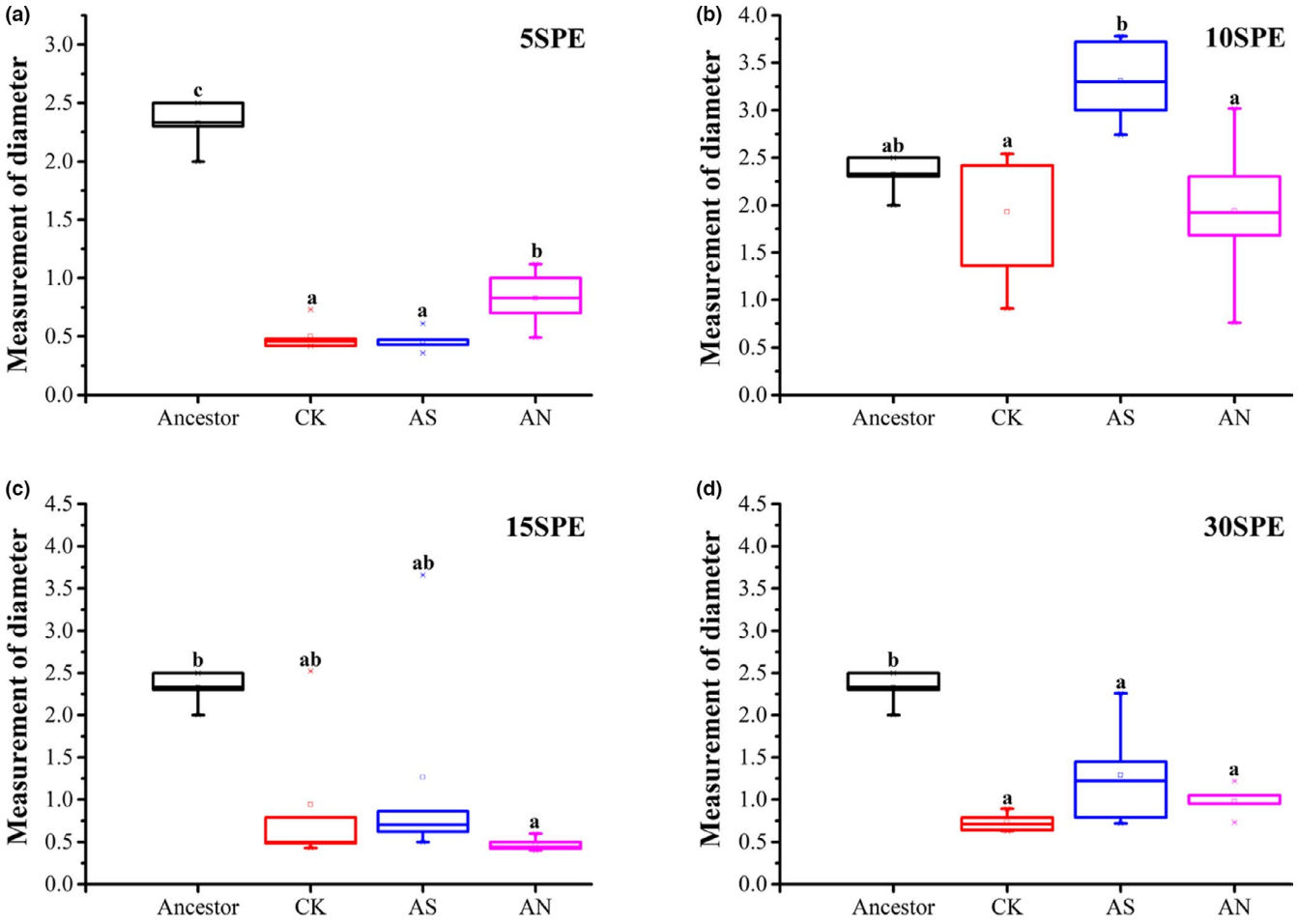

FIGURE 4 Effects of a slight excessive of ammonium sulphate (AS) or ammonium nitrate (AN) on the swimming motility of *Ralstonia solanacearum*. (a) Difference in the swimming motility in five serial passage experiment (SPE) of each treatment. (b) Differences in the swimming motility in 10 SPE of each treatment. (c) Difference in the swimming motility in 15 SPE of each treatment. (d) Difference in the swimming motility in 30 SPE of each treatment. Lowercase letters indicate significant differences ($p < .05$) [Colour figure can be viewed at wileyonlinelibrary.com]

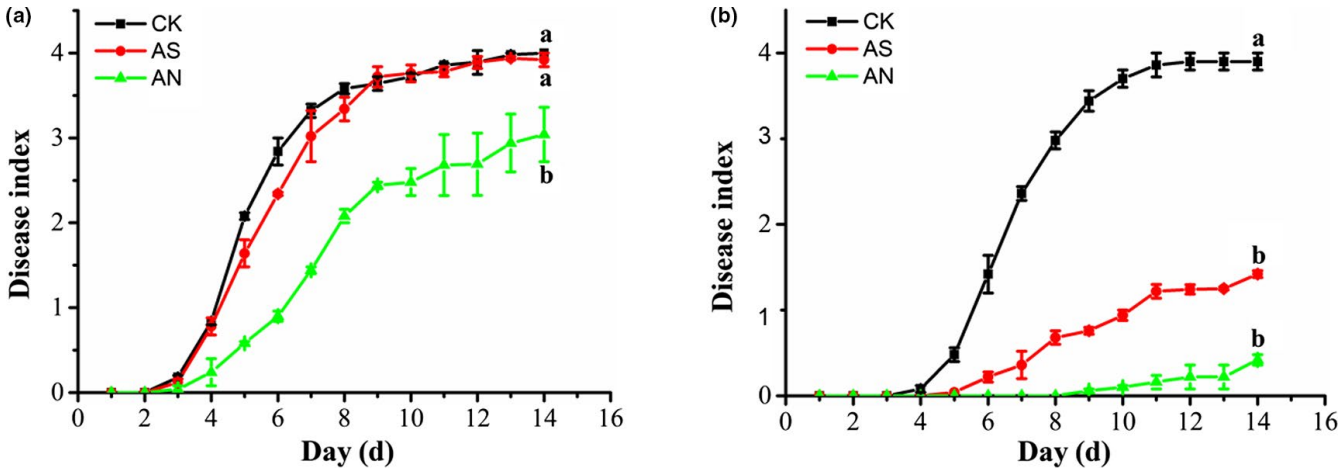

FIGURE 5 Effects of a slight excessive of ammonium sulphate (AS) or ammonium nitrate (AN) on the disease index of *Ralstonia solanacearum*. (a) Difference of disease index in five serial passage experiment (SPE) of different treatments. (b) Difference of disease index in 10 SPE of different treatments. Lowercase letters indicate significant differences ($p < .05$) [Colour figure can be viewed at wileyonlinelibrary.com]

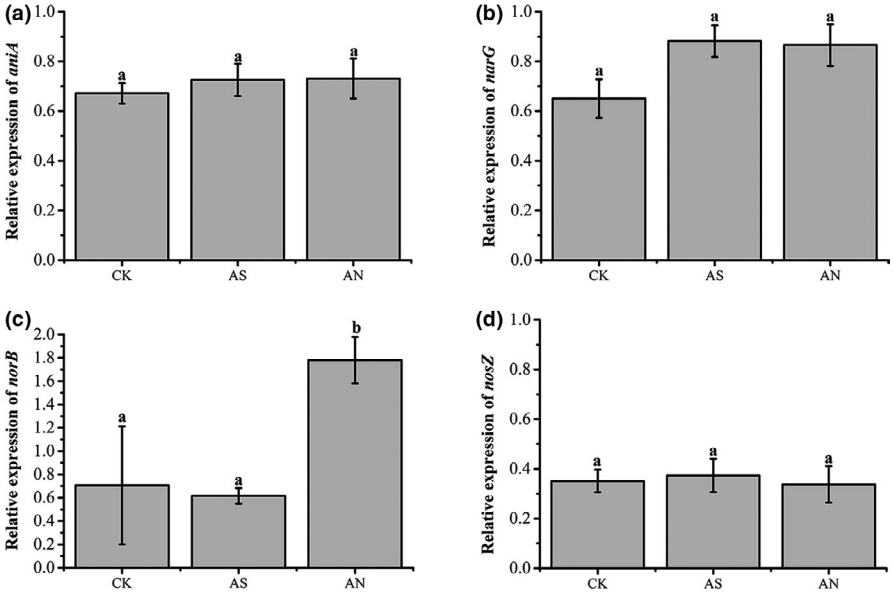

FIGURE 6 Difference in the relative expression of nitrogen metabolism enzyme genes of different serial passage experiment (SPE) strains. Differences in the relative expression of *aniA* (a), *narG* (b), *norB* (c) and *nosZ* (d) in five SPE of each treatment. AS, ammonium sulphate treatment; AN, ammonium nitrate; CK, the control treated without exogenous nitrogen. Lower-case letters indicate significant differences ($p < .05$)

invade quickly when they encounter a suitable host. They could transform into a strong pathogenicity state under the stimulation of plants, resulting in plant disease (Genin, 2010). From these results, we can see that the relationship between nitrogen, soil acidification and *R. solanacearum* needs to be further explored.

In conclusion, the phenotype and pathogenicity of *R. solanacearum* have changed to some extent under the direct effect of nitrogen. The increase of biofilm and swimming mobility, along with the attenuation of pathogenicity, indicated that the bacteria were adapted to the stress environment of excessive nitrogen. This provides some theoretical support for further discussion on the relationship between fertilizer and bacterial wilt. Of course, more evidence about the influence of fertilizer on plant diseases is required.

In most tillage systems around the world, ammonium sulphate and ammonium nitrate are the main compounds of nitrogen fertilizer application, and the accumulation of nitrogen is very severe. Based on our results, the effect of excessive nitrogen on *R. solanacearum* could not be ignored, which suggested that reducing the use of chemical fertilizers was necessary to control the occurrence of plant disease.

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CONFLICT OF INTEREST

All authors declare no competing interests.

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