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Original article – Thematic Issue

Ammonium stress and supplemental calcium influence growth of in vitro cultured blueberry plantlets

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Summary

Background of the study – **Salt damage has recently become a serious problem worldwide. Though ammonium-N is widely used as the main N source for growth of blueberry, few studies on ammonium damage to blueberry were reported.** *Objectives* – **The current research aimed to explore the effects of ammonium stress and supplemental calcium on blueberry growth using an in vitro model.** *Methods* – **In vitro cultured blueberry plantlets subjected to 1/4 MS itself containing 0.005M NH_4^+, 1/4 MS containing 0.01M NH_4^+, 1/4 MS containing 0.02M NH_4^+, 1/4 MS containing 0.02M NH_4^+ plus 0.01M Ca^{2+}. Plant biomass, nutrient compositions and anti-oxidants in plantlets were analyzed.** *Results* – **Ammonium stress induced by 0.02M NH_4^+ significantly inhibited growth of in vitro cultured blueberry. Plants under ammonium stress showed the highest contents of N and Fe, and lowest contents of K, Ca, Mg and Mn. Ammonium stress also significantly inhibited contents of proline (Pro), Methane Dicarboxylic Aldehyde (MDA), soluble protein (SP) and the activity of superoxide dismutase (SOD), and promoted the activities of catalase (CAT), peroxidase (POD) and glutamine synthetase (GS). 0.01M supplemental Ca^{2+} proved to enhance the growth of blueberry plantlets exposed to ammonium stress with the obviously increased contents of K, Ca, Mg and Mn, Pro, MDA, SP and the increased activity of SOD. Close correlations were observed among plant biomass, nutrient compositions and anti-oxidants in blueberry plantlets.** *Conclusions* – **Ammonium stress affects growth of blueberry plantlets by regulating the allocation of nutrient composition and the anti-oxidant activity, which closely correlate with calcium signaling pathways. Supplemental Ca^{2+} could prevent ammonium damage. In vitro culture used as a model provides an effective and promising approach for study of plant stress, and a powerful technical support for field management of *Vaccinium* plants.**

Keywords

ammonium stress, enzyme activity, nutrient composition, plant biomass, supplemental Ca^{2+}

Significance of this study

What is already known on this subject?

- Salt damage has recently become a serious problem throughout the world. Ammonium salt is commonly used as N source for growth of blueberry (*Vaccinium* sp.). Effect of decreased pH induced by ammonium-N on blueberry cultivation was intensively studied, while few studies of ammonium damage to growth of blueberry plants were reported.

What are the new findings?

- Ammonium stress significantly inhibited growth of in vitro cultured blueberry plantlets with the decreased plant biomass by regulating the allocation of nutrient composition and the anti-oxidant activity, which closely correlate with calcium signaling pathways. Supplemental Ca^{2+} could prevent ammonium damage.

What is the expected impact on horticulture?

- In vitro culture used as a model provides an effective and promising approach for study of plant stress, and a powerful technical support for field management of *Vaccinium* plants.

Introduction

Blueberry (*Vaccinium* sp.) belongs to the Ericacease family of perennial deciduous shrubs (Zhao et al., 2012). Due to its richness in polyphenols with a great antioxidant activity, it is of great benefit to our health (You et al., 2011; Zheng et al., 2015). Blueberry is one of the native fruits in the northern hemisphere and widely cultivated commercially, especially in the USA and some European countries (Leitzke et al., 2015). Compared with other crops, the blueberry cultivation has a relatively short history with only about one hundred years (Stribley and Read, 1976). To date, blueberry is becoming one of the most promising crops which are cultivated with a growing interest by the fruit growers worldwide (Brazelton, 2013).

Salt damage has recently developed a serious problem throughout the world (Breś et al., 2016; Maaouiahouimli et al., 2015; Zhao et al., 2016). Growth in response to ammonium stress has often been reported in many plant species, such as grape (Chi and Cormier, 1991), *Arabidopsis* (Li et al., 2014), spinach and pea (Domínguez-Valdivia et al., 2008). N rates are the most important factor contributing to the growth and the yield of blueberry, which have been studied a lot (Banados et al., 2012). Considering ammonium sulfate providing a stable N source and efficiently reducing the soil pH value (Bryla et al., 2008; Ferrari et al., 2015), ammonium sulfate is often used as one of the granular fertilizers in the blueberry cultivation (Bryla and Strik, 2015; Vargas and Bryla, 2015). Blueberry prefers ammonium-N (NH_4^+) over nitrate-N (NO_3^-) (Leitzke et al., 2015; Machado et al., 2012). When the fertilizers are applied with high rate, it can poten-

tially lead to salt damage and thus significantly decrease the blueberry yield (Machado et al., 2012). However, few studies of ammonium damage to growth of blueberry plants were reported.

Conventional methods for the blueberry propagation are time-consuming and restricted by its seasonal growth, which seriously hampers the mass production of the plants. Compared with seedlings, in vitro culture is time-saving and easy to control growth condition of plants. The tissue culture propagation techniques provide us an effective system for the production of virus-free plants (Meiners et al., 2007; Ostrolucká et al., 2004). Of course, in vitro culture can be influenced by many factors, such as plant growth regulators (Cappelletti et al., 2016). To date, in vitro culture technique has been widely used in various aspects and many plant species (Kuo et al., 2015; Zhang et al., 2016a). Considering the merit of in vitro culture, the study was conducted by using in vitro cultured plantlets as a model to analyze the response of blueberry to ammonium stress.

In this work, plant biomass nutrient composition and enzyme activities in blueberry were measured. Supplemental Ca^{2+} was used to further explore the potential mechanism of blueberry plantlets under ammonium stress.

Materials and methods

Plant materials and growth conditions

The plant subject used for in vitro culture is blueberry (*Vaccinium corymbosum* L. 'Bluecrop'). The explants of blueberry were harvested from the one-year old branches before buds germinated in spring and then cultured on solid 1/2 MS (Murashige and Skoog) medium containing 7 g L^{-1} agar, 30 g L^{-1} sugar, 2.2 uM 6-BA and 0.5 uM NAA (Tian et al., 2015). In this study, after the shoot proliferation was stabilized in 1/2 MS, all tested in vitro cultured blueberry were transferred in elongation medium (1/4 MS liquid medium without plant growth regulators). All plantlets were preserved at 23°C for a 16-h light (200 µmol s^{-1} m^{-2}) / 8-h dark period of time at the Plant Tissue Culture Center of Dalian University of Technology.

Experiment design

A preliminary experiment was carried out (Figure S1). Blueberry plantlets cultured with 1/4 MS containing 0.02M NH_4^+ proved an obvious dwarf phenotype. Supplemental Ca^{2+} proved to be effective to prevent ammonium damage when added in 1/4 MS with an appropriate concentration (Wright et al., 1992, 1994). $CaCl_2 \cdot 2H_2O$ were selected as subjects in this study.

In this study, the adventitious buds of blueberry, approximately 1.5 cm long, collected from the 1/2 MS medium described above were separately transferred to the modified 1/4 MS liquid medium without plant growth regulator and with pH 5.0. All plantlets were given four treatments, including 1/4 MS itself containing 0.005M NH_4^+, 1/4 MS containing 0.01M NH_4^+, 1/4 MS containing 0.02M NH_4^+, 1/4 MS containing 0.02M NH_4^+ plus 0.01M Ca^{2+}. $(NH_4)_2SO_4$ was used as the source of NH_4^+. All liquid media were replaced once a week. Each treatment had 9 bottles and every 16 blueberry plantlets were cultured in a glass bottle (height of 9 cm and diam-

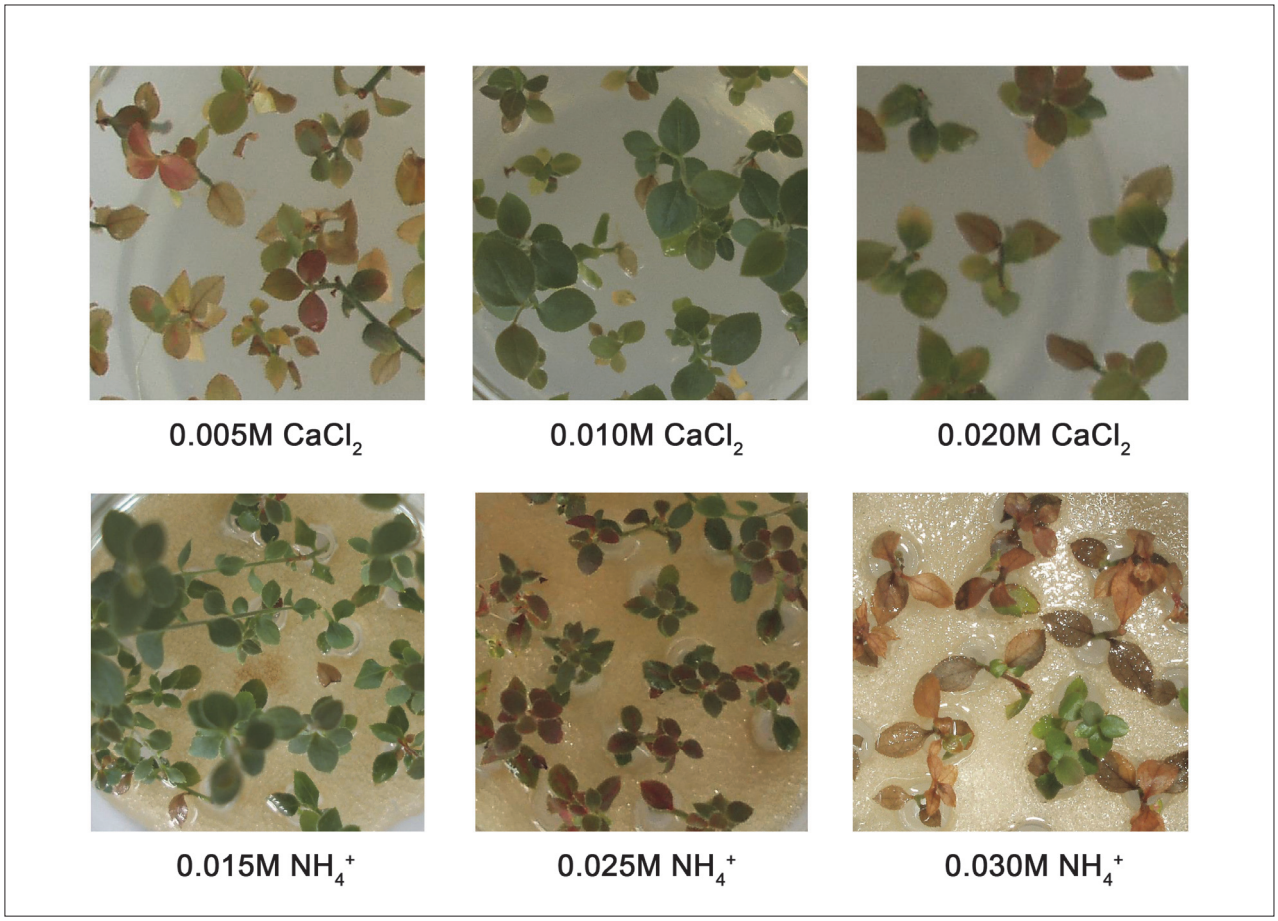


Figure S1. In vitro cultured blueberry plantlets under different treatments.

eter of 5 cm). Plantlets were fixed on a circular bubble which was used as the support and had 16 sterilized tips (Figure S2). All processes were performed under sterile conditions. Plant biomass including plant dry weight, height, chlorophyll contents were measured after cultivation for 60 days. Nutrient compositions and enzyme activities were measured, as well.

Determination of plantlets properties and nutrient compositions in leaves

Every plantlet from three bottles of each treatment was selected to determine the plant biomass (height and dry weight). All samples were washed with deionized water, heated in an oven with 105°C for 30 minutes, and 80°C to constant weight. The total chlorophyll was extracted from 0.2 g Fresh Weight (FW) of leaf tissue in an acetone : dimethyl sulphoxide (DMSO) (1:1 v/v) mix. The supernatant was made up to 1 mL using this mix. The absorption was recorded at the 663 nm and the 645 nm using a UV-visible spectrophotometer Model DU800 (Shimadzu Corporation, Kyoto, Japan). The total chlorophyll was measured as described previously (Porra, 2002) and measured as the percentage reduction comparing to the corresponding control.

For the measurement of nutrient composition, a 0.2 g DW leaf sample was used. The N content was measured with the Kjeldahl method. The Vanadate-molybdate-yellow colorimetry was used to determine the P content. The available K, Ca, Mg, Mn, Fe, Zn and Cu contents were measured using an atomic absorption spectrophotometer (Model Hitachi Z-5000, Hitachi, Tokyo, Japan).

Measurement of enzyme activities

All enzyme extractions were performed at 4°C. All materials sampled at the same time within a day weighing 0.2 g of FW were thoroughly homogenized in a 2.0 mL of 50 mM phosphate buffer (pH value of 7.8) containing 0.1 mM of ethylenediaminetetraacetic acid (EDTA) and 0.1 g of quartz to aid the homogenization. The homogenate was centrifuged at 13.000× *g* for 15 min at 4°C and the crude supernatant was used for further analysis. Contents of proline (Pro) and Methane Dicarboxylic Aldehyde (MDA) were measured by acid ninhydrin method and thiobarbituric acid method, respectively (Walker, 2009). Contents of soluble protein (SP) content was measured using the coomassie brilliant blue G-250 dye-binding method (Qu et al., 2006). The activity of catalase (CAT) was measured using ultraviolet spectrophotometry and the activity of peroxidase (POD) was measured by guaiacol method (Zhang et al., 2014). The activity of superoxide dismutase (SOD) was measured using the nitroblue tetrazolium (NBT) reaction (Alikamanoglu et al., 2011). The activity of glutamine synthetase (GS) was measured using ultraviolet spectrophotometry (Silveira et al., 2003), as well.

Data analysis

A statistical analysis was performed using SPSS (version 17.0). Inter-group comparisons were analyzed using the one-way analysis of variance (ANOVA) followed by a Duncan test. Correlation analysis was done among plant biomass, nutrient compositions and the antioxidants. Before analysis, normal distribution was carried out. Error bars represent ± SE of nine replicates. Different letters above the bars indicate significantly different values ($P < 0.05$).

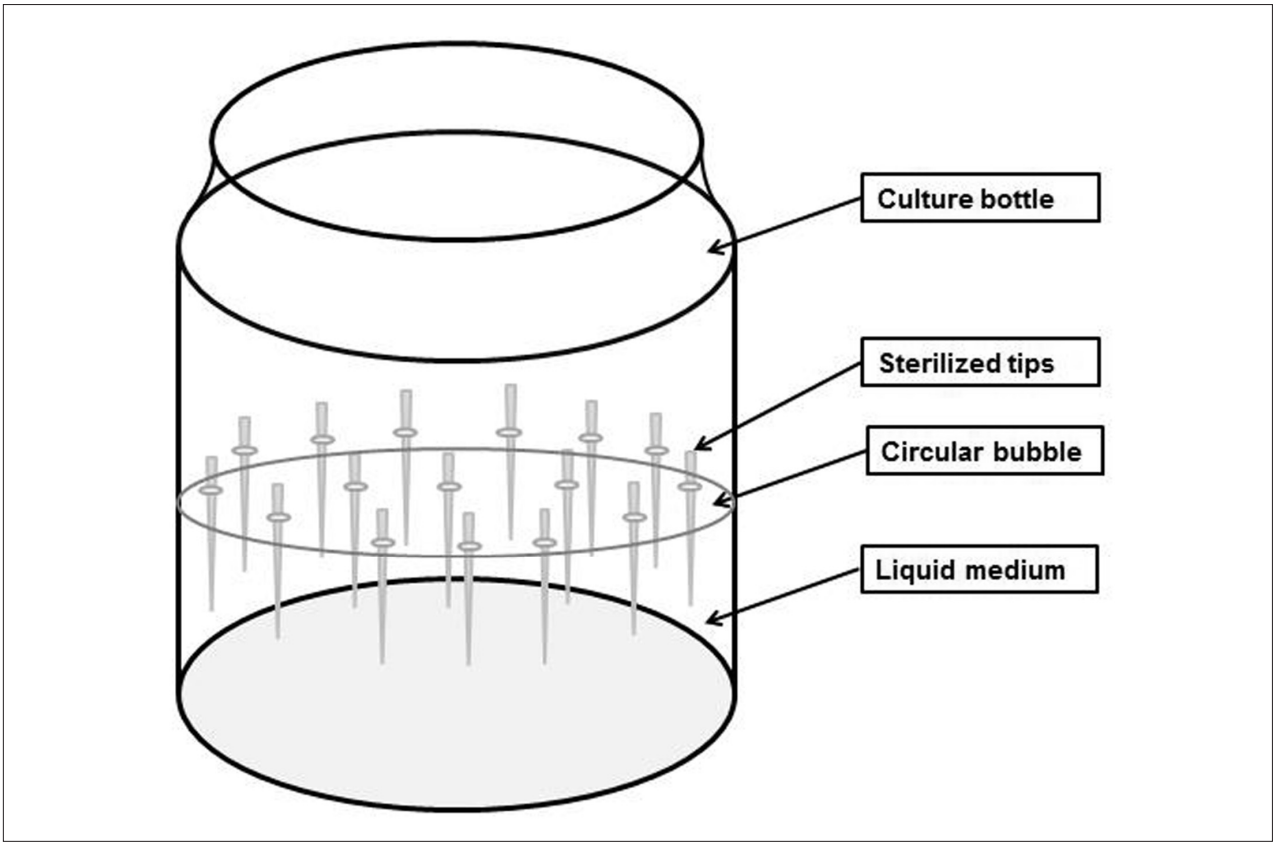


Figure S2. A sketch for in vitro culture.

Results

Ammonium stress and supplemental calcium affects growth of blueberry plantlets

Different phenotypes were observed in blueberry plantlets after the 60 days of exposure to ammonium (Figure 1). The growth of blueberry plantlets was inhibited by 0.02M NH_4^+, which developed into ammonium stress. Compared with lines of 0.005 NH_4^+, 0.01M NH_4^+ obviously promoted the blueberry plantlets growth. Whereas, 0.03M NH_4^+ was lethal for blueberry plantlets (Figure S1). The plant biomass, including plant height, dry weight, chlorophyll *a* and *b* contents, were all in consistence with the phenotype variations. Interestingly, ammonium stress significantly promoted chlorophyll *a*/*b* in 0.02M NH_4^+ lines compared to lines treated with 0.005M NH_4^+ or 0.01M NH_4^+ ($P < 0.05$).

Compared with plantlets of 0.02M NH_4^+, supplemental 0.01M Ca^{2+} significantly improved blueberry growth with the higher plant biomass in blueberry plantlets ($P < 0.05$). Moreover, supplemental Ca^{2+} promoted the accumulation of chlorophyll content, evidenced by the fact that significantly higher chlorophyll contents were observed in plantlets than that of 0.02M NH_4^+ ($P < 0.05$).

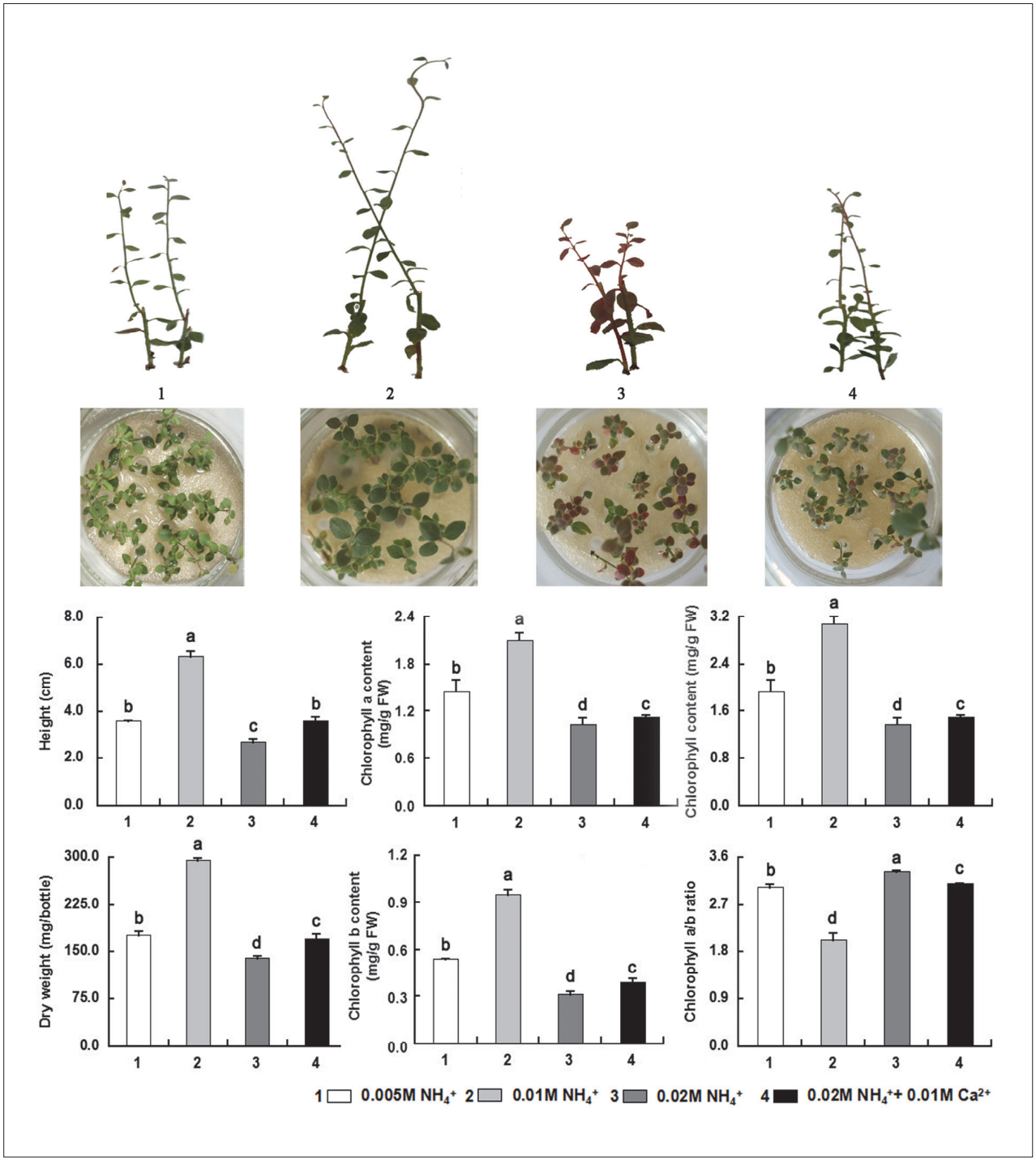


Figure 1. Phenotypes of *Vaccinium corymbosum* L. 'Bluecrop' blueberry plantlets. Different letters above the bars indicate significantly different values ($P < 0.05$).

Responses of nutrient compositions in blueberry leaves to ammonium stress and supplemental calcium

Contents of nutrient compositions, including six macro-elements and three micro-elements, were measured (Figure 2). As expected, the N absorption was increased with the increasing N rates. The N contents in lines of 0.02M NH_4^+ were higher than the lines of 0.005 and of 0.01M NH_4^+ ($P < 0.05$). Ca contents in blueberry plantlets were dramatically increased after supplemental Ca^{2+}. Compared with plantlets under ammonium stress, supplemental Ca^{2+} markedly reduced N, P and Fe contents, but significantly enhanced K, Mg and Mn contents in blueberry plantlets ($P < 0.05$). In addition, ammonium stress induced by 0.02M NH_4^+ and supplemental Ca^{2+} had little effect on Na and Zn contents.

Close correlations were also observed among plant biomass and nutrient compositions (Table 1). The total chlorophyll contents were positively correlated with the height (r = 0.97, $P = 0.001$), the dry weight (r = 0.98, $P = 0.001$), the contents of P and K (r = 0.65, $P = 0.001$; r = 0.75, $P = 0.001$), but negatively correlated with the Chlorophyll a/b (r = -0.98, $P = 0.001$).

Table 1. Correlation analysis of plant biomass and nutrient compositions.

	Height	Dry weight	Total chlorophyll	Chlorophyll *a*/*b*
Height	1.00			
Dry weight	0.99**	1.00		
Total chlorophyll	0.97**	0.98**	1.00	
Chlorophyll *a*/*b*	-0.99**	-0.99**	-0.98**	1.00
N	-0.19	-0.19	-0.31	0.18
P	0.68**	0.70**	0.65**	-0.71**
K	0.64**	0.66**	0.75**	-0.65**
Na	-0.03	-0.05	-0.06	0.01
Ca	-0.28	-0.30	-0.26	0.32
Mg	0.18	0.20	0.30	-0.19
Fe	-0.09	-0.07	-0.07	0.05
Mn	-0.24	-0.24	-0.11	0.24
Zn	0.17	0.19	0.23	-0.20

Note: ** indicates significant at the $P < 0.01$ level.

Analysis of the anti-oxidant activity in blueberry

As shown in Figure 3, contents of proteins Pro, MDA, and SP as well as enzyme activities of CAT, SOD, POD and GS in blueberry leaves were measured. Ammonium stress induced by 0.02 NH_4^+ significantly inhibited the expression of Pro, MDA and SP and the activity of SOD, which showed the same general profile. On the contrary, the activities of CAT, POD and GS in blueberry plantlets were induced by ammonium stress, which was significantly higher than that in lines of 0.005 NH_4^+ and 0.01 NH_4^+ ($P < 0.05$). Supplemental Ca^{2+} significantly promoted the expression Pro, MDA and SP and the activity of SOD ($P < 0.05$). Meanwhile, compared with plantlets of 0.02 NH_4^+, 0.01M supplemental Ca^{2+} reduced the activities of CAT, POD and GS in plantlets ($P < 0.05$).

In addition, correlation analysis showed that the contents of Pro (r = 0.72, $P = 0.001$), MDA (r = 0.91, $P = 0.001$), and SP

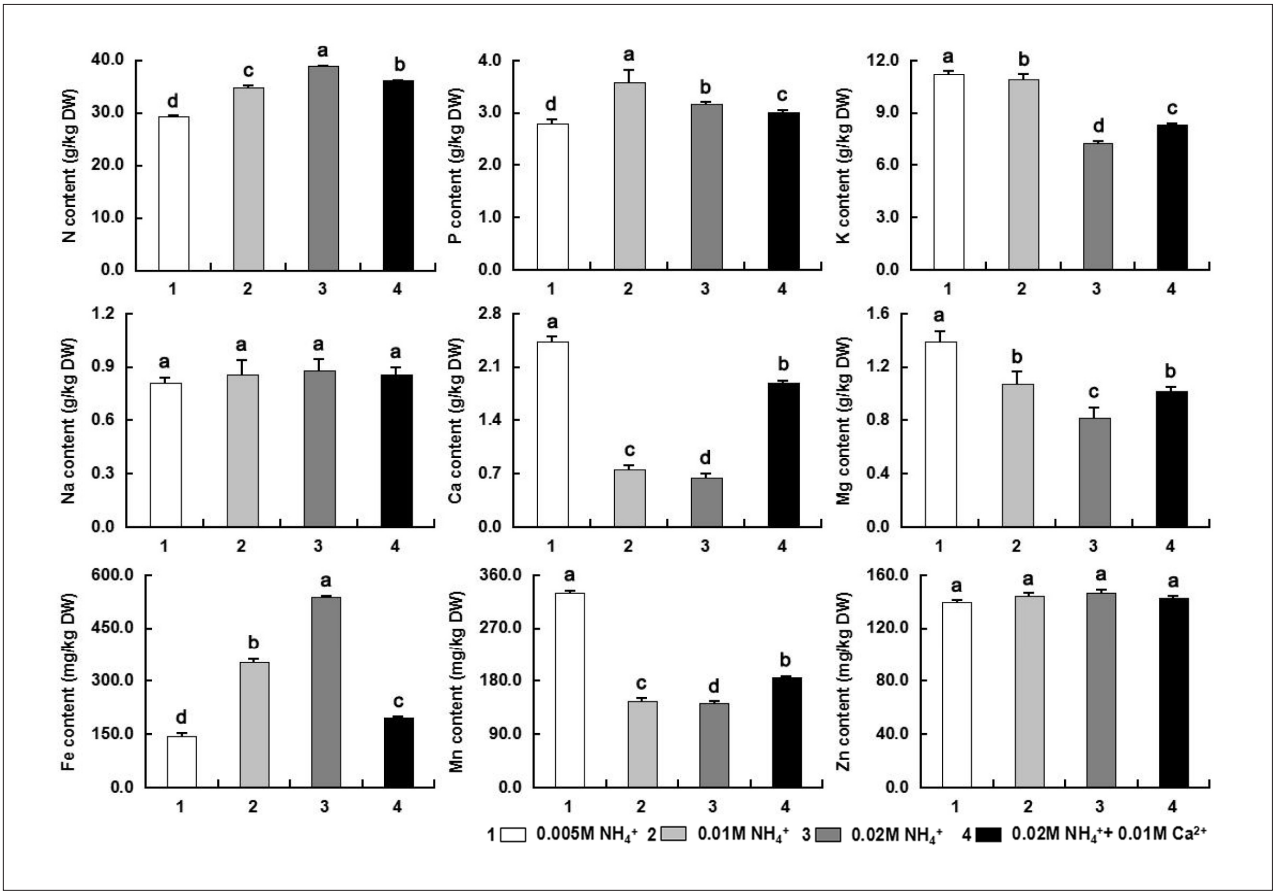


Figure 2. Nutrient compositions in leaves of *Vaccinium corymbosum* L. 'Bluecrop' blueberry plantlets. Different letters above the bars indicate significantly different values ($P < 0.05$).

Table 2. Correlation analysis of Pro contents and MDA, SP contents, and enzyme activities.

	Pro	MDA	SP	CAT	SOD	POD	GS
Pro	1.00						
MDA	0.80**	1.00					
SP	0.73**	0.90**	1.00				
CAT	-0.56**	-0.79**	-0.82**	1.00			
SOD	0.72**	0.91**	0.83**	-0.80**	1.00		
POD	-0.42*	-0.76**	-0.79**	0.84**	-0.77**	1.00	
GS	-0.50**	-0.80**	-0.83**	0.85**	-0.80**	0.91**	1.00
Chlorophyll *a*	0.40*	0.76**	0.79**	-0.85**	0.81**	-0.94**	-0.92**
Chlorophyll *b*	0.421*	0.74**	0.79**	-0.87**	0.76**	-0.94**	-0.92**
N	0.05	-0.29	-0.41*	0.44**	-0.47**	0.58**	0.51**
Ca	0.05	0.04	0.14	-0.1	0.22	-0.20	-0.16

Note: * indicates significant at the $P < 0.05$ level, and ** indicates significant at the $P < 0.01$ level.

(r = 0.83, $P = 0.001$) were positively correlated with the activity of SOD, and that all of them were negatively correlated with the activity of POD, CAT and GS in blueberry plantlets ($P < 0.01$) (Table 2).

Discussion

Due to its potential medical and economic value, blueberry is intensively studied. $(NH_4)_2SO_4$ is commonly used as ammonium-N source for growth of blueberries (Bryla and Strik, 2015). However, NH_4^+ proves a paradoxical nutrient ion, which can result in toxic symptoms in many plants, especially when NH_4^+ is used as the exclusive N source (Britto and Kronzucker, 2002; Machado et al., 2012). Although intensive work has been carried out to unravel the causes and mechanisms of the NH_4^+ toxicity in plants (Britto and Kronzucker, 2002), the present knowledge is far from complete and relatively little is known about the effect of ammonium stress in blueberry plants. In this study, an in vitro model was used to explore the effects of ammonium stress and supplemental calcium on blueberry.

Previous studies proved that growth of blueberry can be significantly affected by different fertilizer with three levels (Li et al., 2012b; Yadong et al., 2009). Ammonium-N is efficient to reduce the soil pH value (Bryla et al., 2008; Ferrari

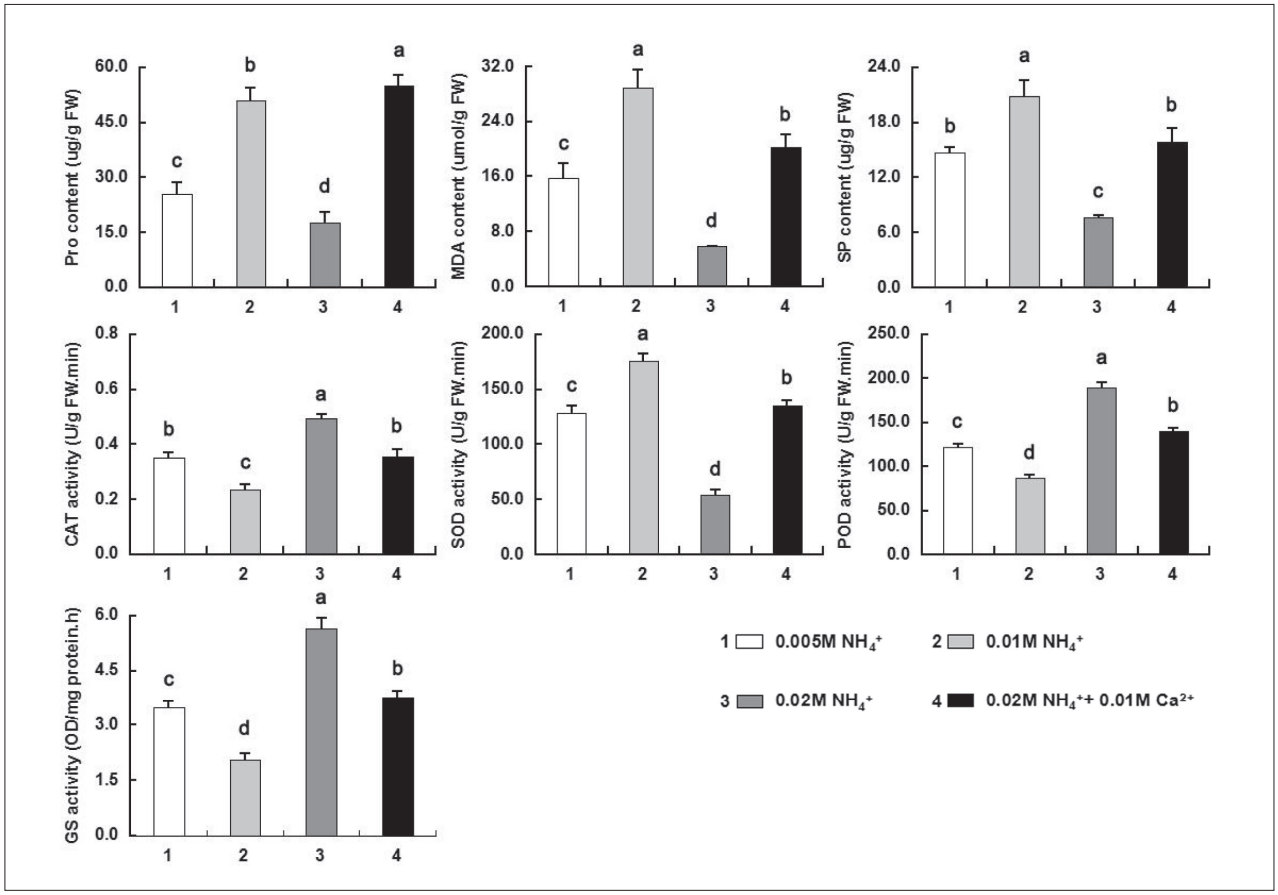


Figure 3. Analysis of anti-oxidant activity in *Vaccinium corymbosum* L. 'Bluecrop' blueberry plantlets. Different letters above the bars indicate significantly different values ($P < 0.05$).

et al., 2015), which is an important factor influencing blueberry cultivation (Poonnachit and Darnell, 2004). In our study, the growth of blueberry plantlets varied greatly with different NH_4^+ concentrations. To eliminate the effect of pH, liquid media with pH 5.0 was replaced once a week, during which pH value was measured twice. The measure proves to be effective to keep pH value basically unchanged and avoids the other effects of unchanged media with long time on plant growth. Unlike field production, in vitro culture provides us a feasible approach to screen new blueberry cultivars with a higher pH value tolerance (Tsuda et al., 2014), which must be helpful to better manage blueberry cultivation in high-pH soils. The results revealed that ammonium stress induced by 0.02M NH_4^+ significantly inhibited the blueberry growth and 0.01M NH_4^+ significantly promoted the plant growth. Plant biomass, including plant height, dry weight, contents of chlorophyll *a* and contents of chlorophyll *b* were all decreased in plantlets under ammonium stress. Interestingly, ammonium stress resulted in a significant increase in the chlorophyll *a*/*b* but a significant decrease in the total chlorophyll. The result may be due to faster degradation of the chlorophyll *b* than chlorophyll *a*. Chlorophyll *b* can be degraded by chlorophyll *b* reductase under high-light conditions (Sato et al., 2015). Ammonium stress may affect chlorophyll *a*/*b* reductase activity and thus regulated chlorophyll contents in our study.

Analysis of nutrient compositions in blueberry plants demonstrated that ammonium stress significantly reduced P, K, Ca, Mg and Mn contents, and promoted N and Fe contents in blueberry plantlets. However, little effect was observed on Na contents in plantlets, which was different with a previous study (Muralitharan et al., 1992). Under ammonium stress, the increasing Na^+ contents was accompanied with a loss of K^+ in leaves of highbush blueberry (Muralitharan et al., 1992). The paradoxical result may be due to the different materials in this study and previous study. On the other hand, different nutrient compositions in plants may be influenced by different stress. Under cadmium stress, the absorptions of K, Ca, Mg, Fe, Cu, and Zn were seriously inhibited in the perennial ryegrass (Wang et al., 2013). Effect of nutrient-elements varies on growth of plant and the response of various nutrient-elements changes greatly under the same stress (Amling, 1960). In our study, $(NH_4)_2SO_4$ was used as the N-source for blueberry development and $CaCl_2$ was alleviator for the ammonium damage. Although some studies revealed that element S may affect growth of blueberry (Spiers and Braswell, 1992) and Cl^- may significantly affect growth of plant, for example, cucumber (Liu and Ma, 2000), the effect of SO_4^{2-} or Cl^- may be potential to blueberry or negligible (Muralitharan et al., 1992; Wright et al., 1992). Our findings reveal that in vitro cultured plantlets are more sensitive to ammonium stress. Further study is essential to explore potential effects of various nutrient-elements on growth of blueberry.

As a second messenger, calcium is involved in many plant signaling processes. Studies have proved that Ca^{2+} mediate salt stress signaling pathway by binding the downstream protein such as calmodulin (Schmitt et al., 1988). Calcium is an essential nutrient that regulates the plant growth and development and many plant hormones involve it (Li et al., 2008). In our study, 0.01M supplemental Ca^{2+} alleviated the damage induced by ammonium stress. Blueberry plantlets showed decreased N, P and Fe contents and increased K, Ca, Mg and Mn contents. Exogenous $CaCl_2$ reduces salt stress in sour jujube by reducing Na^+ and increasing K^+, Ca^{2+}, and Mg^{2+} in different plant organs (Jin et al., 2016). Chlorophyll *a* and *b* contents were both increased in plantlets after supplemental Ca^{2+}. However, chlorophyll *a*/*b* was decreased. Fe proved to be closely correlated with chlorophyll biosynthesis (Zhang et al., 2016b). A study revealed that 79 differently expressive protein spots in Florida Hybrid Bunch Grape under water stress, involved in metabolism, photosynthesis, signal transduction, response to stimulus, calcium signaling pathway, anti-oxidative systems and related to epigenetic modification (Ramesh, 2016). Our result reveals that Ca^{2+} signaling pathway may involve in photosynthesis and nutrient compositions allocation under ammonium stress.

Correlation analysis demonstrated close correlation between plant biomass and nutrients compositions. Given the specific functions of P for protein synthesis for all enzymatic activities (Reich and Oleksyn, 2004), it is not surprising that plant biomass of blueberry correlates with P contents. However, plantlets under ammonium stress showed the highest N content and the lowest Ca content. The plant biomass in blueberry with 0.02M NH_4^+ was significantly smaller than the lines with 0.005M NH_4^+ and 0.01M NH_4^+. In a 6-year field experiment with highbush blueberries, application of $(NH_4)_2SO_4$ helped increase N but decrease Ca in leaves in 3 of the 6 years, showing an invert tendency between contents of Ca and N (Townsend, 1973). Meanwhile, no correlations were observed between Ca and plant biomass. The effect of ammonium stress induced by 0.02M NH_4^+ to blueberry is long-term and potential. Though supplemental Ca^{2+} significantly promoted plant biomass, the regulatory mechanism in plant may be still disordered. 0.01M supplemental Ca^{2+} prevents ammonium damage with only an increase in K, Ca, Mg and Mn contents, but a significant decrease in N, P and Fe contents. Our findings suggested that the excessive accumulation of N contents in leaves is harmful to the plant development and significantly influences Ca^{2+} signaling pathway. The potential ways of such influence that could occur are complex.

Any physiological stress can result in an excessive accumulation of peroxides and a degeneration of cellular membranes in the plant. In the present study, we measured the contents of Pro, MDA, SP and the enzyme activities of SOD, POD, CAT, GS in blueberry, which played vital roles in defending stress (Chong et al., 2015). The low Pro, MDA, SP contents and SOD activity were induced by ammonium stress, together with the high POD, CAT and GS activities in plantlets. Relative analysis demonstrated that Pro, MDA, and SP contents were positively correlated with SOD activity, but negatively correlated with POD, CAT and GS activities. The excessive NH_4^+ may initially promote GS activity and destroy the plant's physiological environment, which further results in the decreased SP contents and the varied anti-oxidative enzymes activities in blueberry plantlets. Changes in all the parameters (SOD, CAT, POD, MDA) in *Vallisneria natans* seedlings exposed to ammonia in combination with microcystin were time-dependent (Ge et al., 2012). For *Spartina alterniflora*, ammonium stress resulted in the high antioxidant enzyme activities of SOD and CAT and the low MDA content (Hessini et al., 2013). Response to ammonium stress varies greatly in different plants. In our study, blueberry under ammonium stress demonstrated the activity of SOD decreased and that of POD and CAT increased. As the first line of defense against reactive oxygen species, SOD mainly scavenges O^{2-}, and POD and CAT mainly scavenges H_2O_2 (Habib et al., 2016). The results above demonstrated that ammonium stress participated in lipid peroxidation and the accumulation of reactive oxygen species, especially H_2O_2.

An appropriate amount of Ca^{2+} is essential for the blueberry development. Calcium plays a vital role in the struc-

ture and function of plant cell membrane. Supplemental 0.01M Ca^{2+} also markedly promoted the accumulation of Pro, MDA and SP in our study, which closely correlated with cell membrane activity. For anti-oxidative enzymes, the activities of SOD were promoted and the activities of CAT, POD and GS were decreased in blueberry plantlets, which suggested that O^{2-} was the main form of reactive oxygen species under mild stress. To be different, supplemental Ca^{2+} promoted the enzyme activities of SOD, CAT, POD, ascorbate peroxidase (APX) and glutathione reductase (GR) and the leaf chlorophyll content in *Brassica juncea* L. under salt stress for 24 hours (Khan et al., 2012). Another study also demonstrated that low concentrations of the supplemental Ca^{2+} may prevent the damage to rabbiteye blueberry (*Vaccinium ashei* Reade) and high concentrations of the supplemental Ca^{2+} could lead to a metabolic damage in the calcifuge *Vaccinium* species (Wright et al., 1992, 1994). Our findings demonstrated that enzyme activities can be significantly regulated by Ca^{2+} signal pathway. Supplemental 0.01M Ca^{2+} could effectively alleviate the impact of oxidative stress induced by ammonium stress in blueberry plantlets. However, correlation analysis revealed that no correlations were observed between Ca and the anti-oxidant activity. Enzyme activities may be regulated by other different genes (Li et al., 2012a) or cytokines (Cappelletti et al., 2016), as well. The potential mechanism is complex. Further study of the correlation among ammonium stress, Ca^{2+} signal pathway and genes or cytokines is essential to be done.

Conclusion

Our findings provide a powerful technical support for seedling management of *Vaccinium* plants. Ammonium stress inhibits the growth of blueberry plantlets with the decreased plant biomass in regulation of the nutrient compositions allocation and antioxidant enzyme activities. Supplemental Ca^{2+} effectively prevents ammonium damage through modulation of antioxidant enzyme activities and the accumulation of Pro, MDA. Ammonium stress closely correlates with calcium signaling pathways. Importantly, in vitro culture provides an effective and promising approach for the study of plant stress.

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Competing interests

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