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ORIGINAL ARTICLE

Effect of Exogenous Ca^{2+} on Growth and Accumulation of Major Components in Tissue Culture Seedlings of *Sophora tonkinensis* Gagnep

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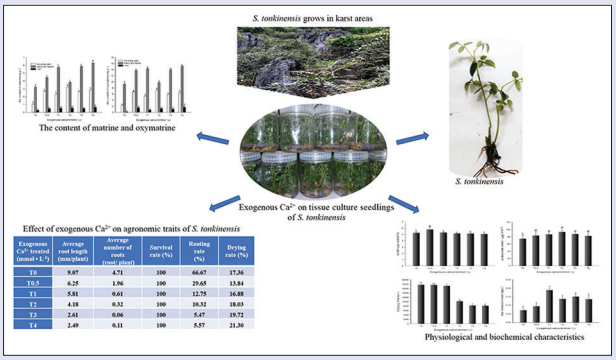
ABSTRACT

Background: *Sophora tonkinensis* Gagnep., well-known for its medicinal properties, grows in karst areas of China characterized by calcium (Ca) and drought. Due to excessive excavation, wild *S. tonkinensis* is almost extinct. Ca regulates the growth and development of plants and also functions in stress response. **Objectives:** The effects of exogenous Ca^{2+} on plant height, stem diameter, enzyme activity, endogenous hormone content, and other traits of *S. tonkinensis*, and accumulation of matrine and oxymatrine in its active constituents were studied. The purpose was to explore the mechanism of adaptation of *S. tonkinensis* to high Ca, to protect and artificially cultivate *S. tonkinensis* resources. **Materials and Methods:** Six concentrations of Ca^{2+} (0, 1.495, 2.99, 5.98, 8.97, and 11.96 mmol/L) were used. Agronomic traits were measured with a ruler, vernier calipers, and an electronic scale. Physiological and biochemical indices were measured using ultraviolet spectrophotometry. Endogenous hormone contents were determined by enzyme-linked immunosorbent assay. We determined the composition of oxymatrine and matrine using high performance liquid chromatography. **Results:** As Ca^{2+} concentration increased, the drying rate of *S. tonkinensis* decreased and then increased; root length and number, and rooting rate decreased; and the soluble sugar, soluble protein, and chlorophyll content increased and then decreased. reactive oxygen species increased, increasing enzyme activity to resist cell membrane damage under low concentrations of Ca^{2+} (0–2.99 mmol/L); high concentrations of Ca^{2+} (5.98–11.96 mmol/L) did more damage to *S. tonkinensis*, and enzyme activity was not coordinated. Low concentrations of Ca^{2+} (0–2.99 mmol/L) promoted methyl jasmonate content to reduce cell damage. Matrine and oxymatrine in the whole plant were highest under 5.98 mmol/L Ca^{2+} treatment, and in stems, leaves, and roots they were highest under 11.96 mmol/L Ca^{2+} treatment. **Conclusion:** *S. tonkinensis* can tolerate exogenous Ca concentrations between 2.99 and 5.98 mmol/L.

Key words: Active components, agronomic traits, calcium, physiological and biochemical characteristics, *Sophora tonkinensis* Gagnep

SUMMARY

- Exogenous Ca^{2+} has a good effect on the accumulation of major components of *Sophora tonkinensis*
- Exogenous Ca^{2+} can increase the drying rate of *S. tonkinensis*
- The redox system of *S. tonkinensis* under proper exogenous calcium was less damaged, making it easy to maintain normal growth through self-regulation and repair.



Abbreviations used: ELISA: Enzyme-linked immunosorbent assay; HPLC: High-performance liquid chromatography; ROS: Reactive oxygen species; MS medium: Murashige and Skoog medium; NAA: A-naphthalene acetic acid; IBA: Indole-3-butyric acid; BSA: Bovine serum albumin; Chla: Chlorophyll a; Chlb: Chlorophyll b; SOD: Superoxide dismutase; NBT: Nitro blue tetrazolium; POD: Peroxidase; CAT: Catalase; MDA: Malondialdehyde; ABA: Abscisic acid; BR: Brassinol; GA: Gibberellin; JA-ME: Methyl jasmonate; Z: Zeatin.

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INTRODUCTION

Sophora tonkinensis Gagnep. (family Leguminosae), is a well-known medicinal plant of China that grows in karst areas.[1] Its roots and rhizomes, known as "Shan-Dou-Gen" in Chinese, have historically been used to treat the accumulation of heat toxicity, sore throat, swollen gums, and sore mouth and tongue.[2] More than 150 chemical components, including alkaloids, flavonoids, polysaccharides, volatile

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oils, and small amounts of triterpenoids and phenols, have been isolated from *S. tonkinensis*.[3] Among them, alkaloids, especially matrine and oxymatrine, are the main medicinal active ingredients of *S. tonkinensis*.[4] *S. tonkinensis* is mainly distributed in southern China and North Vietnam.

Long-term deforestation has caused serious damage to wild resources.[5] In recent years, the natural habitat of *S. tonkinensis* has been rapidly destroyed but demand has increased.[6,7] In addition, *S. tonkinensis* is sporadically distributed in the karst terrain of Guangxi, where the environmental conditions are relatively poor due to high calcium (Ca) and drought. For a long time, vegetation with calcareous, lithologic and xerophytic characteristics has developed in karst terrain,[8] including *S. tonkinensis*. Currently, research on *S. tonkinensis* mainly focuses on its clinical efficacy and chemical composition, while there is limited research on calcareous (Ca) stress of *S. tonkinensis*. There have been no studies on the effects of Ca on physiology, biochemistry, endogenous hormones, or the content of medicinal active ingredients of *S. tonkinensis*. Such studies are necessary because karst is a typical calcareous environment. Moreover, Ca is the major macronutrients in plants and is the second messenger that is ubiquitous in plant signaling. Ca is involved in regulating growth and development in plants. It also increases a plant's tolerance for abiotic stressors.[9]

Hence, to find the optimal Ca concentration for *S. tonkinensis*, this study established different Ca^{2+} concentration media to cultivate high-quality strains of *S. tonkinensis*. The agronomic traits, physiological and biochemical indexes, and the content of medicinal active ingredients in tissue-cultured seedlings of *S. tonkinensis* were determined under different Ca^{2+} concentrations. The effects of varying Ca concentrations on the tissue culture of *S. tonkinensis* were discussed and we proposed a theoretical basis to improve the culture of *S. tonkinensis*.

MATERIALS AND METHODS

Plant material

Tissue culture material of *S. tonkinensis* was collected from Nanning, China. The Guangxi Key Laboratory of Medicinal Resources Protection and Genetic Improvement at Guangxi Botanical Garden of Medicinal Plants identified the original plant.

Ca^{2+} treatment and experimental setup

Tissue culture of *S. tonkinensis* was carried out with different Ca concentration formulas and the agronomic traits, physiological and biochemical indexes, and the content of medicinal active ingredients were determined after the tissue culture seedlings grew for 45 days.

A single factor, 5 level test was used to set the exogenous Ca^{2+} concentration gradient in the medium based on the optimal $CaCl_2$ standard (2.99 mmol/L $CaCl_2$). The gradient included no Ca and Ca values that were half, one time, two times, three times, and four times of the standard, which were T0 (C_{Ca} = 0 mmol/L), T0.5 (C_{Ca} = 1.495 mmol/L), T1 (C_{Ca} = 2.99 mmol/L), T2 (C_{Ca} = 5.98 mmol/L), T3 (C_{Ca} = 8.97 mmol/L), and T4 (C_{Ca} = 11.96 mmol/L), respectively. Sterile seedlings of *S. tonkinensis* were put onto solid Murashige and Skoog medium at half the macronutrient concentration, and contained a sucrose value of 3% w/v and an agar powder value of 0.35% w/v with a gel strength of 1100 g/cm. 0.3 mg/L indole-3-butyric acid and 1.0 mg/L A-naphthalene acetic acid supplemented this. The tissue culture seedlings of *S. tonkinensis* were cultured in an illuminated incubator at, 25°C ± 1°C, and a exposed to light for 16 h/d at an intensity of 1200 lux.[7]

Determination of growth parameters

The number of surviving plants, root length, dry weight, root number, and fresh weight of each bottle of tissue culture seedlings were recorded and the rooting rate, survival rate, drying rate, and average root length were calculated.

Root length was determined using a ruler, stem diameter was determined using vernier calipers, and fresh weight and dry weight were determined using an electronic scale. Plants were rinsed with distilled water to clean them. After they were dry, plants were weighed to determine the fresh weight of the root and the stem.

Determination of soluble sugar content

The anthrone method was used to measure soluble sugar. The absorbance at 640 nm was measured using methanol as a blank. The concentration of soluble sugar was calculated using glucose solution as a standard.[10]

Determination of protein content

Dye-binding was used to determine the total protein content of each extract of enzyme[11] with a protein assay kit (Tiangen Biotech Co., LTD, BJS China).

Determination of photosynthetic pigments

Chlorophyll a (Chla) and Chlorophyll b (Chlb) were extracted using 95% ethanol. The absorbance of the extracts was measured using a Model UV-752N spectrophotometer (Inesa, China) at wavelengths 665, 649, and 470 nm. The contents of Chlb, total chlorophyll (Chla + b), and Chla were measured using Wang and Huang's method.[12]

Determination of enzyme activities

After harvesting, the leaves were immediately frozen in liquid nitrogen. Until the enzyme assays, leaves were stored at −80°C.

Superoxide dismutase (SOD) activity was measured based on its performance inhibiting the photochemical reduction of nitro blue tetrazolium (NBT) at 560 nm. A 1 mL sample of supernatant was added to the reaction mixture (1.8 mL 0.1 mol/L phosphate buffered saline [PBS], 0.3 mL 0.13 mol/L methionine, 0.3 mL 0.75 mol/L NBT, 0.3 mL 0.1 mmol/L ethylenediaminetetraacetic acid-Na_2 and 0.3 mL 0.02 mmol/L of riboflavin). The solution was kept in an artificial climate chamber at 30°C, and the light reaction was started. After 15 min, the light was turned off. This caused the reaction to stop. Afterward, we measured the absorbance at 560 nm.

The increase in absorbance was monitored in the phosphate buffer (pH 7.8) at 470 nm to determine peroxidase (POD) activity when guaiacol was present. A 50 μL sample of supernatant was added into 3 mL POD substrate solution. Immediately after stirring, the absorbance measurement was carried out at a wavelength of 470 nm, and the 0 s and 60 s absorbance values were recorded.

Catalase (CAT) activity was determined according to the method used by Aebi.[13] This was based on the initial reduction rate at 240 nm in H_2O_2. A 0.10 mL sample of supernatant was added into 2 mL 0.05 mol/L PBS and 0.9 mL 0.1 mol/L H_2O_2. After stirring, the we measured absorbance at 240 nm wavelength and the 0 min, 1 min, and 2 min absorbance values were recorded.

Determination of lipid peroxidation

The malondialdehyde (MDA) content was measured using Wang and Huang's method[12] to approximate the extent of cell membrane damage. For this measurement, the control tube contained: 2 mL 0.1 mol/L phosphate buffer, 2 mL 5% (w/v) trichloroacetic acid (TCA) solution and 2 mL 0.67% (w/v) thiobarbituric acid (TBA) solution. The sample tube contained: 2 mL enzyme solution, 2 mL 5% (w/v) TCA solution and 2 mL 0.67% (w/v) TBA solution. The mixture was placed in boiling water for 30 min. The reaction was stopped after the tubes were placed on ice.

The samples were centrifuged, and the absorbance of the supernatant was measured at 450, 532, and 600 nm.

Quantification of plant hormones by enzyme-linked immunosorbent assay

The plant hormones in *S. tonkinensis* under different Ca^{2+} treatments were determined by enzyme-linked immunosorbent assay (ELISA). The plant hormone ELISA kits (abscisic acid [ABA], brassinol [BR], gibberellin [GA], methyl jasmonate [JA-ME] and zeatin [Z]) used here were purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd. The leaves of *S. tonkinensis* were treated according to the product instruction manual. The test was repeated three times.

Determination of matrine and oxymatrine contents

Roots and rhizomes were collected from the *in vitro* rooting culture of tissue culture seedlings after 35 days and further dried and the levels of oxymatrine and matrine were measured according to the guidelines of the Chinese Pharmacopoeia (edition 2015).[2]

RESULTS

Effect of exogenous Ca^{2+} on agronomic traits

With the increase of exogenous Ca^{2+}, the survival rate of tissue culture seedlings of *S. tonkinensis* was still 100% [Table 1]. With increased Ca^{2+} concentration, the root length, rate of rooting and root number of *S. tonkinensis* were significantly reduced. Among them, the rate of rooting and root number in the T3 treatment group was the lowest and the root length in the T4 treatment group was the shortest. With the increase of exogenous Ca^{2+} concentration, the drying rate of *S. tonkinensis* decreased first and then increased gradually relative to T0. The T4 treatment had the highest drying rate, reaching 21.30%, which was 22.7% higher than the control. Thus, increasing the exogenous Ca^{2+} concentration inhibited the growth and development of the roots of *S. tonkinensis* seedlings, but increased its drying rate.

Effect of exogenous Ca^{2+} on physiology and biochemistry

As the Ca^{2+} concentration increased, the soluble sugar content increased first and then decreased. In T0.5 treatment, the soluble sugar content was significantly higher than other treatments and increased by 10.0% compared with T0 treatment to 5.81%. Under the other Ca^{2+} concentration treatments, the soluble sugar content was not significantly different from T0 [Figure 1].

The amount of soluble protein first increased and then decreased as the Ca^{2+} concentration was increased. The soluble protein content was the highest in T2 treatment and increased by 24.6% compared with T0 treatment, increasing to 93.60 μg/g/FW. At T0 treatment, the soluble protein content was at least 75.11 μg/g/FW. The level of soluble protein was not significantly different among different Ca treatments except T2 treatment [Figure 1].

Exogenous Ca^{2+} significantly affected the total chlorophyll content in *S. tonkinensis*. The total chlorophyll content increase first and then decrease as the Ca^{2+} concentration increased. The total amount of chlorophyll was significantly higher in the T1 treatment than in other treatments, which was 2.6 times that of T0 treatment and increased to 0.18 mg/g. In the T2, T3, and T4 treated plants, the amount of total chlorophyll was significantly higher by 85.7%, 114.2%, and 85.7%, respectively, compared to exogenous treatment at T0 [Figure 1].

Exogenous Ca^{2+} reduced the antioxidant enzyme activity of *S. tonkinensis* to some extent. Compared with T0 treatment, the POD activities in T0.5, T1, T2, T3, and T4 treatments decreased by 5.3%, 2.7%, 41.9%, 52.9%, and 53.6%, respectively. SOD activity was reduced by 0.1%, 12.3%, 6.5%, 0.9%, and 3.5%, respectively. In the T1 treatment, the activity of SOD was significantly lower than in T0, T0.5, and T3 treatments. As the Ca^{2+} concentration increased, the CAT activities first increased and then decreased. The activity of CAT in T2 treatment was the strongest, at 30% higher than that in T0 treatment, reaching 311.60 U/g FW/min. Thus, the antioxidant enzyme activities in *S. tonkinensis* showed different trends [Figure 1].

The content of MDA in the T1 treated *S. tonkinensis* was the highest, at 6.3% higher than that of T0 treatment and increased to 0.032 μmol/g. We found no significant difference in the MDA content in *S. tonkinensis* with T0.5, T1, T2, and T0. The content of MDA under T3 and T4 treatment was significantly lower than that of T0 treatment, which decreased by 28.1% and 25.0%, respectively [Figure 1].

The endogenous hormones ABA, BR, Gibberellin A3, JA-ME, and Z were different at different Ca^{2+} concentrations. Under different exogenous Ca^{2+} concentrations, the contents of ABA in *S. tonkinensis* did not significantly differ from the control. The contents of BR, JA-ME, and Z were higher than those of the control under different exogenous Ca^{2+} treatments. When treated with T4, the ABA content was the highest, at 6.3% higher than that of T0 treatment, increasing to 0.84 μg/g. When treated with T2, the ABA content was the lowest, at 5.1% lower than that of T0 treatment and decreased to 0.75 μg/g. In T4 treatment, the BR content was significantly higher than the other treatments, at 57.9% higher than the T0 treatment and increased to 0.30 ng/g. When treated with T0.5, the BR content was second at 0.23 ng/g. When treated with T0.5, the content of GA_3 was the highest, at 20.2% higher than that of T0 treatment, increasing to 35,166.81 pg/g. When treated with T1 and T2, the content of JA-ME was the highest, at 1.0% higher than that of T0 treatment and increased to 5.99 pmol/g. When treated with T0.5, the Z content was the highest, at 14.7% higher than that of T0 treatment and increased to 9.31 pmol/g [Figure 2].

The effect of exogenous Ca^{2+} on accumulation of matrine and oxymatrine

Secondary metabolites play an important role in osmotic adjustment in response to environmental stressors.[14] Here, exogenous Ca^{2+} had a significant effect on the synthesis of matrine and oxymatrine

Table 1: Effect of exogenous Ca^{2+} on agronomic traits of *Sophora tonkinensis*

Exogenous Ca^{2+} treated (mmol/L)	Average root length (mm/plant)	Average number of roots (root/plant)	Survival rate (%)	Rooting rate (%)	Drying rate (%)
T0	9.07	4.71	100	66.67	17.36
T0.5	6.25	1.96	100	29.65	13.84
T1	5.81	0.61	100	12.75	16.88
T2	4.18	0.32	100	10.32	18.03
T3	2.61	0.06	100	5.47	19.72
T4	2.49	0.11	100	5.57	21.30

Average root length (mm/plant): Total root length/total number of plants; Average number of roots (root/plant): Total number of roots/total number of plants; Rooting rate (%): Total number of roots per plant/total number of plants ×100; Drying rate (%): Fresh weight/dry weight ×10

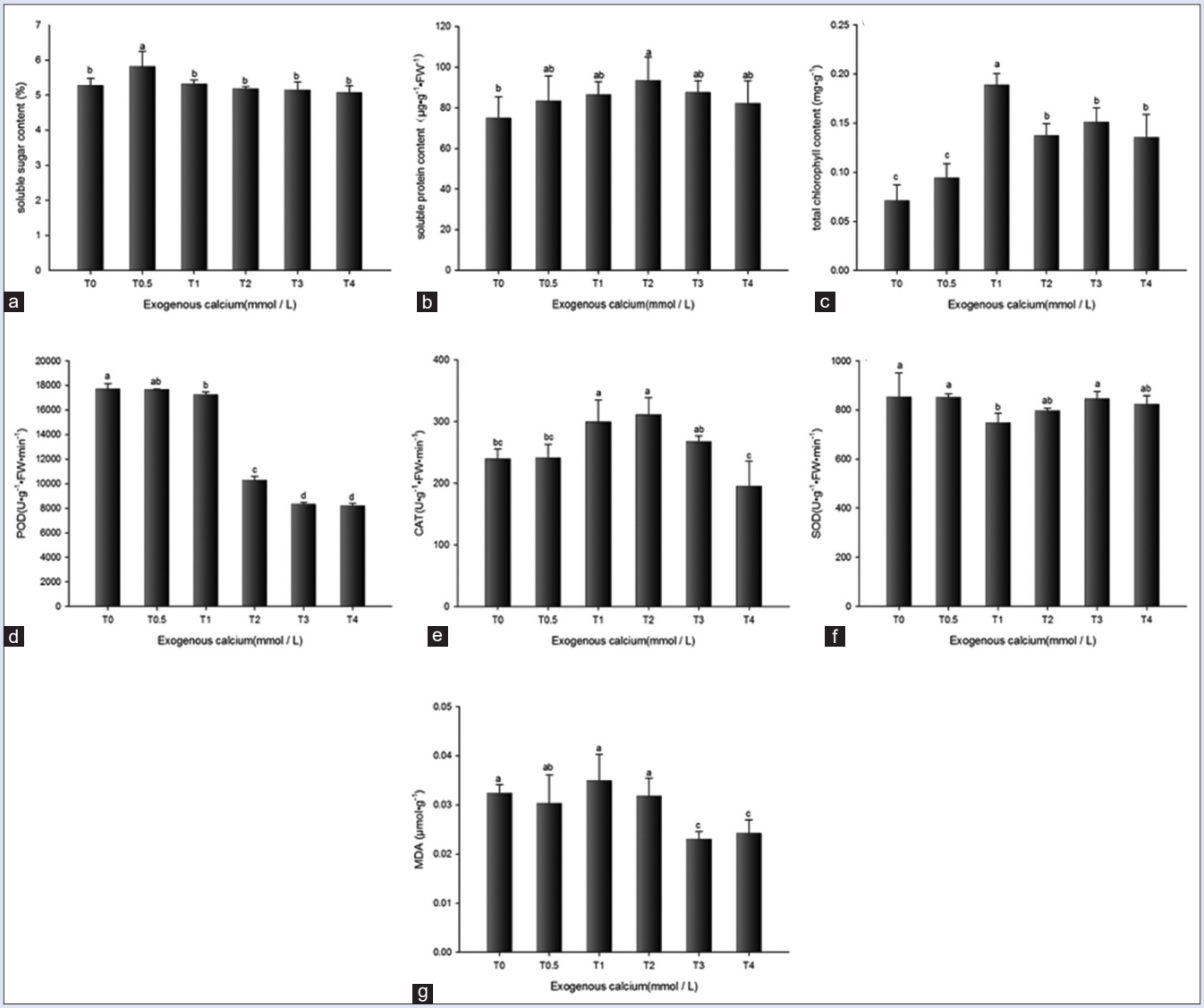

Figure 1: Effect of exogenous Ca^{2+} on (a) soluble sugar content, (b) soluble protein content, (c) total chlorophyll content, (d) peroxidase, (e) catalase, (f) superoxide dismutase, and (g) malondialdehyde. Each bar is the mean ± standard error, $n = 3$. Values followed by the same letter are not significantly different at $P < 0.05$

[Figure 3]. As the concentration of Ca^{2+} increased, content of matrine and oxymatrine in whole plants, stems, leaves, and roots of *S. tonkinensis* showed an upward trend. Among them, the matrine and oxymatrine components were mainly concentrated in the stems and leaves: The root content significantly lower than the content in the leaves and stems.

From the determination of the whole plant, as the concentration of Ca^{2+} increased, matrine and oxymatrine content in the whole plant increased first and then decreased. When treated with T2, the oxymatrine and matrine contents were highest, at 3.1 times that of T0 treatment and increased to 3.23 mg/g and 7.21 mg/g, respectively. In this case, the contents of matrine and oxymatrine were also significantly higher than the T0 treatment and other exogenous Ca^{2+} treatments.

In stems and leaves, with the increase of Ca^{2+} concentration, the contents of matrine and oxymatrine in stems and leaves showed a trend of increasing gradually. When treated with T4, the content of matrine and oxymatrine in stems and leaves was the highest, at 94.2% and 63.7% higher than that of T0 treatment, which increased to 6.31 mg/g and 15.21 mg/g, respectively. In summary, the content of matrine and oxymatrine in stems and leaves was significantly higher in the T4 treatment than with the T0 treatment and other exogenous Ca^{2+} treatments.

In roots, the contents of matrine and oxymatrine were the highest at T4, at 2.4-fold and 10.2-fold higher than T0, respectively, and increased to 0.66 mg/g and 1.74 mg/g, respectively. In T4 treatment, the content of matrine in roots was significantly higher than that under T0, T0.5, and T3 treatments, but there was no significant difference with that under T1 and T2 treatments. At the T4 concentration, the oxymatrine root content was significantly higher than in the T0, T1, T2, and T3 treatments, but not significantly different from that under T0.5 treatment.

DISCUSSION

Agronomic traits reflect the growth of plants, including characteristics of the roots. Plants mainly rely on roots for nutrient absorption. *S. tonkinensis* tissue culture seedlings may be affected by exogenous Ca, hindering root growth, interfering with plant metabolic systems, and

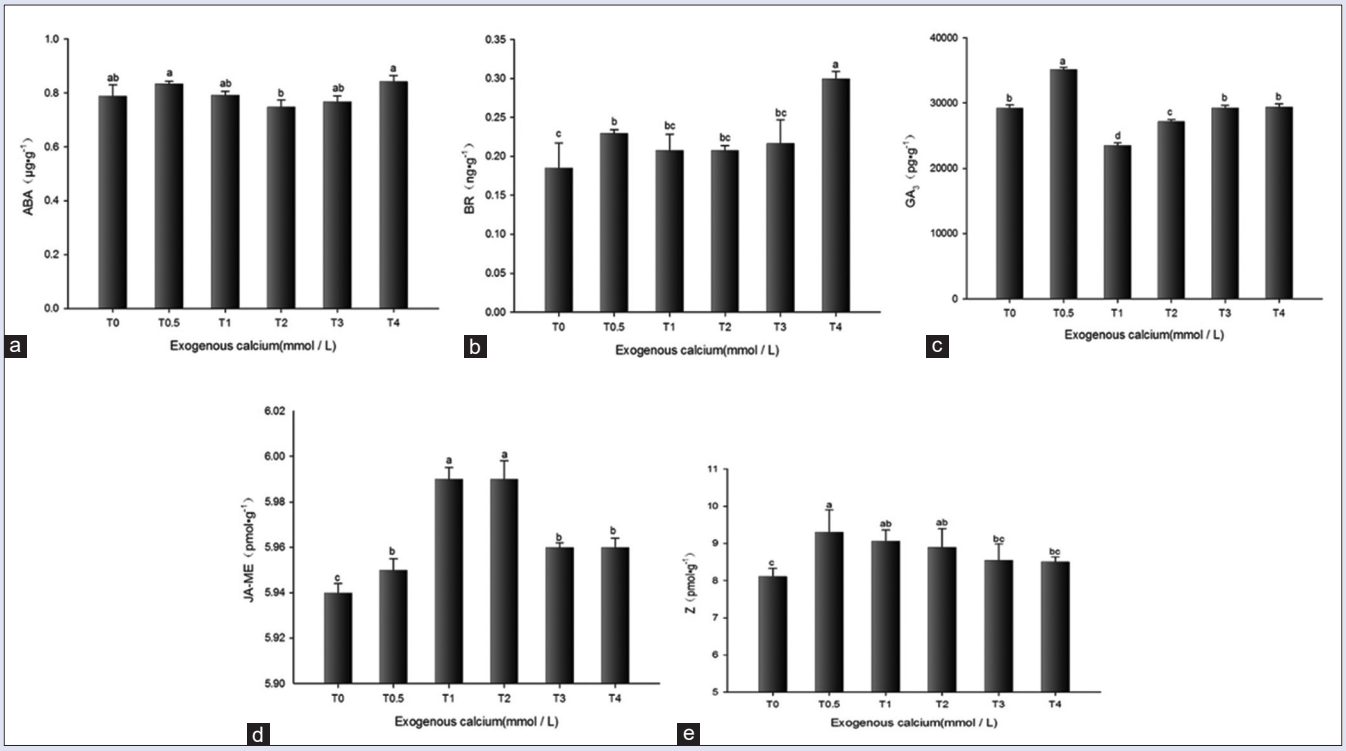

Figure 2: Effect of exogenous Ca^{2+} on (a) abscisic acid, (b) brassinol, (c) GA_3, (d) methyl jasmonate, and (e) zeatin. Each bar is the mean ± standard error, $n = 3$. Values followed by the same letter are not significantly different at $P < 0.05$

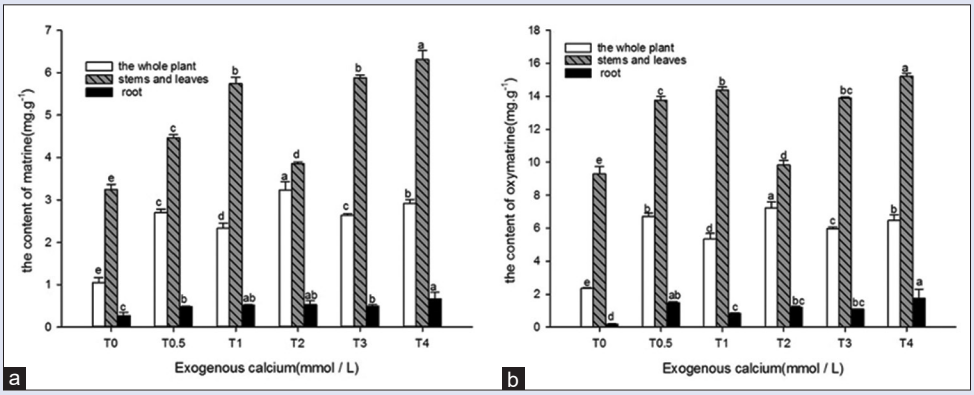

Figure 3: Effect of exogenous Ca^{2+} on (a) the content of matrine and (b) the content of oxymatrine. Each bar is the mean ± standard error, $n = 3$. Values followed by the same letter are not significantly different at $P < 0.05$

accumulating nutrients to inhibit root growth in *S. tonkinensis*. In this study, increased Ca^{2+} concentration resulted in significantly decreased length and number of roots and rooting rate for *S. tonkinensis* cultured seedlings and decreased to the lowest level in T4 treatment. The drying rate, however, increased significantly.

Ca^{2+} is a major element, vital for both the growth and development of a plant, that also regulates the growth and development functions of the stress response of the plant.[15] Exogenous Ca strengthened tolerance of hypoxia in cucumber roots by mediating the movement of Ca^{2+} and K^+ and increasing the accumulation of enzymes related to respiratory metabolism. In order to induce the influx of Ca^{2+} across the plasma membrane, we need reactive oxygen species (ROS) signaling.[16] ROS comes from the aerobic cells of metabolism. They include singlet oxygen ($^1O_2$), hydroxyl-free radical (OH), and hydrogen peroxide (H_2O_2). ROS scavengers involved in enzymatic activities in plants include SOD, POD, CAT, and ascorbate peroxidase.[17]

POD can scavenge H_2O_2 in cells. The activity of POD under T0.5, T1, and T0 treatment was basically the same and was at the highest level, indicating that low Ca (0 mmol/L – 2.99 mmol/L) treatment increased the ROS in *S. tonkinensis*, to reduce the harm of calcareous stress. The activities of POD in T2 to T4 treatments were significantly lower than that under T0 treatment, indicating that the high Ca^{2+} (5.98 mmol/L – 11.96 mmol/L) environment caused greater damage to *S. tonkinensis* so that the enzyme activity could not be coordinated and the lack of consistent enzyme activity made it impossible to resist external stressors. Hence, the activity of POD of *S. tonkinensis*

had certain adaptability to changes in external Ca^{2+} concentration and enhanced POD enzyme activity by self-regulation to combat low calcareous stress.

CAT is a stress-inducing factor in plants and is involved in the regulation of redox in cells. When treated with T2, CAT activity was relatively high. With higher Ca^{2+} concentration, CAT activity decreased significantly, indicating that under low Ca, *S. tonkinensis* can resist damage by increasing CAT activity. However, the redox system of plants under high Ca treatment was greatly damaged, making it difficult to maintain normal growth through self-regulation and repair.

The main function of SOD is to remove superoxide free radicals produced by stress. There were no significant differences between T0.5, T2, T3, T4, and T0 treatments, indicating that plants were damaged by low Ca (0 mmol/L – 2.99 mmol/L) and high Ca (5.98 mmol/L – 11.96 mmol/L). Plants could resist cell membrane damage by increasing SOD activity. The activity of SOD in T1 treatment was the lowest, indicating that T1 treatment had the lowest degree of cell damage.

Soluble sugar plays an important role in regulating plant growth, development, and gene expression because it is one of the most active carbohydrates.[18] In our study, T0.5 treatment had the highest sugar content, indicating that low Ca^{2+} (0 mmol/L – 2.99 mmol/L) may damage the membrane system of *S. tonkinensis*. This could be due to the high level of SOD and POD activity observed in the T0.5 treatment group. *S. tonkinensis* regulates soluble sugar content to provide energy and osmotic pressure of cells to resist damage due to low environmental Ca.

Soluble protein is an important component of enzymes in many plants, which contribute to the development and growth of plants by providing nutrients and energy. Prior research has demonstrated the variety of environmental stresses that can change the gene expression of plants, thus promoting the synthesis of new proteins.[19] It may be that for *S. tonkinensis* cultured in an *ex vivo* library, there is no temperature or moisture stress and thus, the soluble protein content of *S. tonkinensis* does not change much.

Chlorophyll is the most important and most effective pigment in the plant photosynthetic process, and it is also an important factor in determining the photosynthetic rate and dry matter accumulation.[20] In our experiment, the chlorophyll content of *S. tonkinensis* treated with T1 (2.99 mmol/L) was the highest, indicating that the Ca^{2+} concentration of 2.99 mmol/L provided the leaves of *S. tonkinensis* seedlings with an advantage in capturing light energy. Therefore, it was speculated that there was a greater possibility of increasing photosynthetic rate and ultimately increasing yield.

A common indicator to assess lipid peroxidation is MDA content, low levels of which have been found with increased resistance in.[21] The MDA content in the treatments of T3 and T4 was significantly lower than the MDA content of the control, indicating that the high Ca^{2+} (5.98 mmol/L – 11.96 mmol/L) environment greatly damaged *S. tonkinensis* so that the enzyme activity was not consistent and the plants could not resist external stress. Under low Ca (0 mmol/L – 2.99 mmol/L) culture, *S. tonkinensis* can protect against cell membrane damage by increasing enzyme activity, similar to the results of Zhang *et al.*[22] This result indicates that the increase of POD activity, SOD activity, and soluble sugar content protects against damage to the cell membrane under low Ca culture and removes excess ROS.

During stress response, the primary function of ABA is to inhibit stomatal openings, regulate plant transpiration, and promote the accumulation of secondary metabolites.[23] In our study, ABA content in *S. tonkinensis* was the highest in T4 treatment. The levels of oxymatrine and marine in stems, roots, and leaves were significantly higher than they levels in T0 and other exogenous Ca^{2+} treatments.

JA-ME is a signaling molecule of the defense system that stimulates the plant's protective system to reduce cell damage.[24] Low Ca (0 mmol/L – 2.99 mmol/L) promoted the content of JA-ME in *S. tonkinensis,* and high Ca (5.98 mmol/L – 11.96 mmol/L) inhibited the content of JA-ME. Therefore, the cell membrane damage increased with the increase of Ca^{2+} concentration in *S. tonkinensis* from 0 mmol/L – 2.99 mmol/L.

Z can promote the accumulation of sugar and the activity of enzymes, delaying the effect of aging.[25] Low Ca treatment (0 mmol/L – 2.99 mmol/L) promoted Z content, while high Ca treatment (5.98 mmol/L – 11.96 mmol/L) did not promote the content of Z. Thus, Z significantly promoted the accumulation of sugar, and the activity of SOD in T0.5 treated *S. tonkinensis* improved the antioxidant capacity of *S. tonkinensis* and delayed aging.

The content of active ingredients in medicinal plants is one of the criteria for evaluating the quality of medicinal materials.[26] Studies of medicinal plants are different from those of nonmedicinal plants because in addition to considering the agronomic traits and stress resistance, it is also necessary to consider the active ingredient content of medicinal plants. Oxymatrine and matrine levels in the whole plant were highest under T2 treatment. The oxymatrine and matrine levels in stems, leaves, and roots of *S. tonkinensis* were the highest under T4 treatment. These results indicate that the increase of Ca concentration can promote the accumulation of matrine and oxymatrine in *S. tonkinensis*.

CONCLUSION

As Ca^{2+} concentration increased, the drying rate of *S. tonkinensis* initially decreased and then increased, however, the root length, root number, and rooting rate tended to decrease. In addition, as Ca^{2+} concentration increased, the soluble sugar, soluble protein, and chlorophyll content increased first and then decreased. ROS in *S. tonkinensis* increased in order to increase the activity of enzymes to resist cell membrane damage under low concentrations of Ca^{2+} (0–2.99 mmol/L), while high concentrations of Ca^{2+} (5.98–11.96 mmol/L) did more damage to *S. tonkinensis* so that the activity of enzymes could not be coordinated. Similarly, low concentrations of Ca^{2+} (0–2.99 mmol/L) promoted JA-ME content in *S. tonkinensis*, stimulating the plant protection system to reduce cell damage. The contents of matrine and oxymatrine in the whole plant were the highest under 5.98 mmol/L Ca^{2+} treatment, and the content of matrine and oxymatrine in stems, leaves, and roots of *S. tonkinensis* was the highest under 11.96 mmol/L Ca^{2+} treatment. Stems and leaves were enriched with matrine and oxymatrine from *S. tonkinensis*.

After considering the physiological, biochemical, and growth indexes, as well as changes in the content of matrine and oxymatrine of *S. tonkinensis*, the concentration of exogenous Ca to *S. tonkinensis* is suitable between 2.99 and 5.98 mmol/L.

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Conflicts of interest

There are no conflicts of interest.

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