

Inhibitory Effects of Oyster Shell on the Spathe "Greenback" of *Anthurium andraeanum* Lind.

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Abstract *Anthurium andraeanum* Lind. is an important tropical flower. However, the spathe of *A. andraeanum* develops a "greenback" under low-light conditions. This study aimed to evaluate the use of oyster shell supplementation as a cultivation technique for inhibiting the spathe "greenback" of *A. andraeanum* under low-light conditions. Appropriate calcium from micro-dissolution of oyster shell can improve the activity of phenylalanine ammonia lyase (PAL) which is positively correlated with the anthocyanin content. Using regression models and response surface methodology (RSM), the relationships between oyster shell fragments and the anthocyanin content were determined. The results showed that the solubility of oyster shells increased with the increase in fragment weight, C, and time, T at pH 5.9. In oyster shell substrate, cultivation of *A. andraeanum* under low-light conditions (less than $220 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at pH 5.9, regression analysis showed that the PAL activity in pedicels of *A. andraeanum* first increased and then decreased with the increase in oyster shell fragment weight C, and the 286 mg oyster shell fragments greatly increased the PAL activity of *A. andraeanum* pedicels within 8 weeks (w). The 286 mg oyster shell fragments significantly increased the anthocyanin content in *A. andraeanum* spathes under weak-light conditions within 8 h, and the soluble sugar content reached the maximum value at 15 weeks as well, together with the pedicel diameter, soluble sugar and water content, thus inhibiting the spathe "greenback" of *A. andraeanum*.

1 Introduction

Anthurium andraeanum Lind. is an important tropical flower variety [1,2]. Current research shows that the decrease of light intensity caused by excessive shading or prolonged low temperature and rainy periods in bagasse facility cultivation will lead to the occurrence of "greenback" symptoms in the spathe in which a red to green color gradually extends from the base of the spathe to the tip [3], and the quality of flowers is significantly reduced [4].

Anthocyanin is known to be an extremely important chemical component in red flowers, and its content is also one of the key factors determining the color of *A. andraeanum* spathes. Considerable research has proven that the activity of PAL is positively correlated with the content of anthocyanin, and calcium can replace light and hormones to promote PAL activity [5]. Higaki et al. found that the greenback phenomenon was highly correlated with the calcium content in *A. andraeanum* plants, and appropriate exogenous calcium supplementation could significantly alleviate the symptoms [6,7].

Oyster shells, massively deposited on the seashore, are a solid waste byproduct of the mariculture industry and are composed of more than 90% calcium carbonate,

which is not easily dissolved under natural conditions, thus providing a valuable Ca resource [1]. Calcium carbonate can provide a good source of exogenous Ca in the soilless cultivation of *A. andraeanum* and can be used in the supplement [8].

Calcium carbonate, the main component of oyster shells, is a potential long-term exogenous calcium material that micro-dissolves at a suitable pH value and can achieve a similar effect of calcium supplementation. An appropriate pH value and oyster shell fragment size are key to overcome the greenback symptoms of *A. andraeanum* under low-light conditions.

The study of oyster shells supplementation of Ca in the cultivation of *A. andraeanum* has not been previously investigated. This paper aimed to test the use of oyster shells supplementation of Ca for inhibiting the spathe greenback of *A. andraeanum* by (1) determining the effect of different sizes of oyster shell fragments, which lead to Ca micro-dissolution and increased PAL activity; (2) evaluating the effects of oyster shell fragment mass and cultivation time on the pedicel diameter, Ca^{2+} content and PAL activity in the pedicels; and (3) determining the changes in the soluble sugar, water content, and anthocyanin content in the spathe with oyster shell supplementation.

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2 Materials and methods

2.1 Materials

Three-year-old *A. andraeanum* 'Tropical' was grown in a 5-L flow aquarium using a general nutrient solution for *A. andraeanum* supplemented with oyster shell fragments (one plant per box). Five-centimeter-long flower branches were obtained with no mechanical damage and no pests or diseases, from which the pedicels were used for the determination of Ca, PAL and pedicel diameter, while the spathe was used for determination of the anthocyanin content, the soluble sugar content and water content determination. The oyster shells were immersed in 0.1% hydrochloric acid solution for 10 h, and then the oyster shells were dried naturally. The oyster shells were crushed using a grinder and then screened. Finally, the required oyster shell fragments were obtained.

2.2 Method

2.2.1 Dissolution test method of oyster shell with different weights treated for different times

Based on a previous test result (pH 5.9, (Xia & Chen, 2019), 31.25, 62.5, 125, 250, and 500 mg) oyster shell fragments were selected as experimental materials and were soaked in 5-L phosphoric acid buffer flow aquariums at pH 5.9. The residual weight of the oyster shell was measured regularly (every 1 h, 3 h, 5 h, 7 h, 9 h) and recorded separately. Solubility of oyster shells under different weight, C, and time, T, treatments was recorded and repeated three times.

2.2.2 Test method for cultivation of *A. andraeanum* with 286 mg oyster shell fragments as a substrate

In 286-mg oyster shell fragment soilless culture of *A. andraeanum* with a general nutrient solution at pH 5.9 [9], the Ca content, PAL activity, diameter of the pedicel, and anthocyanin, soluble sugar and water contents of the spathe were determined and repeated three times under low-light conditions^[5] ($220 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at different times (4, 6, 8, 10, 12 weeks).

2.2.3 Oyster shell supplementation effect on PAL and flower quality of *A. andraeanum*

The oyster shell fragments were combined with the *Anthurium* general nutrient solution (the nutrient solution was changed once a week). The extraction and determination of PAL used the method of OUYANG G.C. (1985) [10]. The supernatant was measured at OD₂₉₀ with an ultraviolet spectrophotometer. The determination of the anthocyanin content followed the method of the East China Normal University Biological Department (1980) [11]. The filtrate was filtered and measured spectrophotometrically at OD₅₃₀. The relative concentration unit of the anthocyanins was expressed as a

factor of 10. Extraction and determination of soluble sugars followed the anthrone-sulfuric acid method. Determination of the water content was based on the drying method.

2.2.4 Data analysis

The data were processed using Microsoft Excel 2013 and were analyzed using SPSS Statistics 20.0 (IBM Corp., Armonk, NY, USA). Using the response surface methodology (RSM), the second-order polynomial equation $y=b_0+b_1x_1+b_2x_2+b_3x_1x_2+b_4x_1^2+b_5x_2^2$ was used to analyze the results of each index in a complex factor and multi-level test. The fitted polynomial equation was then illustrated using three-dimensional surface plots to visualize the relationships between the responses of the variables.

3 Results and Analysis

3.1 Effects of Fragment Weight, C (mg), on the Dissolution Time of Oyster Shells

The dissolution tests of oyster shells at different weights, C, of 31.25, 62.5, 125, 250 and 500 (mg) were carried out at a pH value of 5.9. The solubility of oyster shells of different weights, C, at different treatment times, T, was determined as shown in Table 1.

Table 1. Solubility analysis of oyster shell (OS) fragments with different weights, C (mg), treated for different times, T (h)

OS Content C (mg)	1 h	3 h	5 h	7 h	9 h
31.25	0.023	0.025	0.025	0.026	0.025
62.5	0.028	0.027	0.028	0.029	0.028
125	0.031	0.033	0.032	0.034	0.034
250	0.036	0.036	0.037	0.036	0.037
500	0.045	0.047	0.047	0.048	0.047

According to the regression analysis in Table 1, as shown in Figure 1, the solubility of oyster shells increased with the increase in fragment weight, C, and time, T. The larger the fragment (weight) and the longer the time, the higher the solubility. When C=855.2619 (mg) and T=5.8351 (h), the solubility of the oyster shell reached a maximum value of 0.0519 ± 0.0041 (mg). The results showed that the solubility of the oyster shell differed significantly with the fragment weight C and time T ($F=64.02^{**}$, $F_{0.01}=4.1708$, $F>F_{0.01}$).

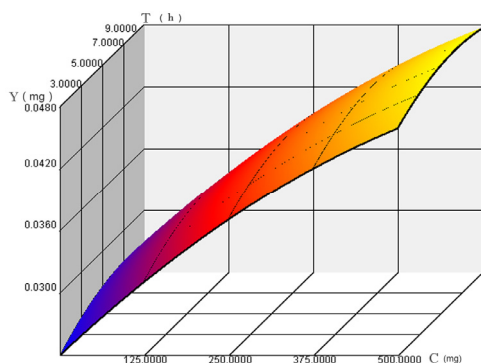


Figure 1. Solubility of oyster shell fragments treated with different weight C (mg) and time T (h)

$y_{\text{dissolution}} = 0.02149 + 0.00006405C + 0.00102T - 0.000000267CT - 0.0000003653C^2 - 0.00006786T^2$ (F=64.02**)
 When C=855.2619 (mg), T=5.8351 (h), the solubility reached a maximum value of 0.0519 ± 0.0041 (mg).

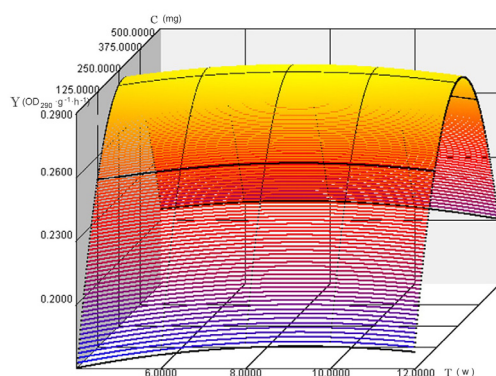


Figure 2. Effects of oyster shell fragments quality C (mg) and time T (w) treatment on PAL ($OD_{290} \cdot g^{-1} \cdot h^{-1}$) activity of the pedicle in *Anthurium*

$y_{\text{PAL}} = 0.1169 + 0.006827T + 0.00104C - 0.000003328TC - 0.0003607T^2 - 0.000001772C^2$ (F=4.1232*)
 When T=8.1438 (w), C=286.07 (mg), the content of PAL reached a maximum of 0.2936 ± 0.0838 ($OD_{290} \cdot g^{-1} \cdot h^{-1}$).

3.2 Effects of oyster shell cultivation on PAL activity in pedicels of *A. andraeanum*, with different C (mg) and T (w) treatments

The activity of PAL in pedicels of *A. andraeanum* cultivated under low-light conditions ($220 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) was determined by applying the general nutrient solution of the oyster shell substrate culture. The effects of C (mg) and time T (w) treatments on the activity of PAL in pedicels were studied.

Regression analysis showed that with the increase in oyster shell fragment weight C, PAL activity in pedicels of *A. andraeanum* increased first and then decreased (Fig. 2). When C=286 (mg) and T=8.1 (w), the activity of PAL reached the maximum value of 0.2936 ± 0.0838 ($OD_{290} \cdot g^{-1} \cdot h^{-1}$); when C exceeded 286 mg, the activity of PAL decreased if the time were prolonged. The possible reason was that the longer the time, the higher the

cumulative calcium content, which would inhibit the activity of PAL, resulting in a decrease in the PAL activity. Statistical analysis showed that there were significant differences in PAL activity between the effects of the oyster shell fragment weight C and time T (F=4.1232*, $F_{0.05}=2.7401$, $F > F_{0.05}$).

3.3 Determination of Related Index Contents of the Flower Quality of *A. andraeanum*

The effects of 286 mg oyster shell fragments on the flower quality of *A. andraeanum* cultivated under low-light conditions ($220 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) at pH 5.9 were compared by measuring the change in the diameter of the pedicels, the water content, the soluble sugar and the anthocyanin content in spathes of *A. andraeanum* (Table 2).

Table 2. Effect of T (w) treatment at different times on the quality of *A. andraeanum* flowers

	4 w	6 w	8 w	10 w	12 w
Pedicel diameter (cm)	0.28	0.32	0.36	0.38	0.38
Soluble sugar ($\text{mg} \cdot 100 \text{g}^{-1} \text{Fw}$)	0.312	0.386	0.453	0.514	0.537
Anthocyanin (OD_{530})	0.680	0.675	0.754	0.783	0.874
Water content (%)	0.84	0.81	0.77	0.80	0.78

3.3.1 Changes in pedicel diameter of *A. andraeanum* cultivated with oyster shell

The regression analysis in Table 2 showed that with the increase in T, the effect of T (w) treatment at different times on the pedicel diameter of *A. andraeanum* increased initially and then slowly decreased. The experimental results in Figure 3 show that when $r=0.9969$, there is a highly significant relationship between the pedicel diameter and the treatment time (F=163.5**, $F_{0.01}=99.00$, $F > F_{0.01}$), indicating that the increase in the pedicel diameter with the increase in time was very significant, but when T=11.64 (w), the pedicel diameter had a maximum value of 0.3819 ± 0.0077 (cm).

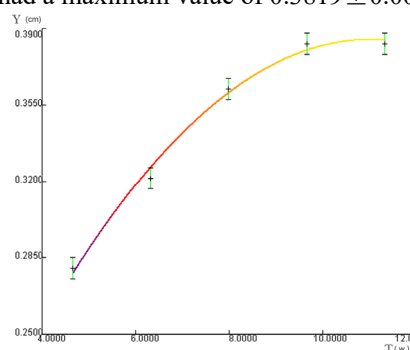


Figure 3. Changes in pedicel diameter under *A. andraeanum* cultivated with oyster shell

$y_{\text{pedicel diameter}} = 0.14 + 0.0415T + 0.00178T^2$ (F=163.5**)
 When T=11.64 (w), the pedicel diameter reached the maximum of 0.3819 ± 0.0077 (cm).

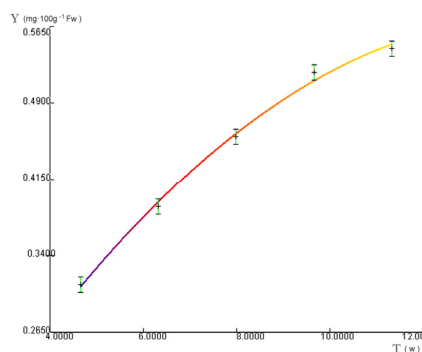


Figure 4. Changes in soluble sugar content in spathe of *A. andraeanum* cultivated with oyster shell

$$y_{\text{sugar}} = 0.101 + 0.0597T \pm 0.00192T^2 \quad (F=306.6^{**})$$

When $T=15.5$ (w), the soluble sugar content reached the maximum value of 0.5641 ± 0.0120 ($\text{mg} \cdot 100\text{g}^{-1} \text{Fw}$).

3.3.2 Changes in the Soluble Sugar Content in Spathes of *A. andraeanum* Cultivated with Oyster Shell

The regression analysis in Table 2 showed that the effect of different times on the soluble sugar content in the spathe of *A. andraeanum* showed an increasing trend with the increase in time T. The results in Figure 4 show that when $r=0.9983$, the soluble sugar content in the spathe has a very significant relationship with the treatment time ($F=306.6^{**}$, $F_{0.01}=99.00$, $F>F_{0.01}$), indicating that the soluble sugar content of the spathe increased significantly with the increase in time, but when $T=15.4926$ (w), the soluble sugar content of the spathe had a maximum value of 0.5641 ± 0.0120 ($\text{mg} \cdot 100\text{g}^{-1} \text{Fw}$).

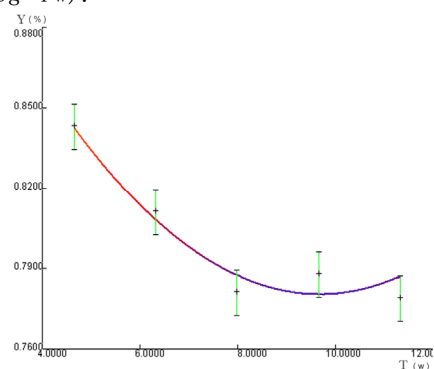


Figure 5. Changes of water content in spathe of *A. andraeanum* cultivated with oyster shell

$$y_{\text{water content}} = 0.942 - 0.0322T + 0.0016T^2 \quad (F=3.1)$$

When $T=10.02$ (w), the water content had the minimum value of 0.7806 ± 0.0306 (%).

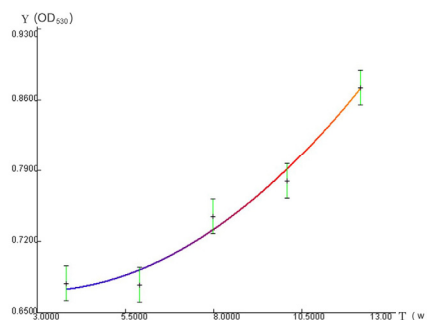


Figure 6. Effects of T (w) treatment on anthocyanin OD₅₃₀ content of spathe in *A. andraeanum*

$$y_{\text{anth}} = 0.6967 \pm 0.01577T + 0.002535T^2 \quad (F=28.5261^{**})$$

When $T=3.1099$ (w), the content of anthocyanin was 0.6723 ± 0.0342 (OD₅₃₀).

3.3.3 Changes in the Water Content in the Spathe of *A. andraeanum* Cultivated with Oyster Shell

The regression analysis in Table 2 showed that the effect of the T (w) treatment at different times on the water content of the *A. andraeanum* spathe showed a downward trend with the increase in the treatment time. The results in Figure 5 show that when $r=0.8695$, the water content of the spathe had no significant relationship with the treatment time ($F=3.1$, $F_{0.05}=19.00$, $F<F_{0.05}$), indicating that the water content of the spathe did not increase significantly with the increase in time. However, when $T=10$ (w), the water content of the spathe had a minimum value of 0.7806 ± 0.0306 (%), when the water content was the lowest, the sugar content increased significantly, and the pedicel diameter approached the maximum value, while the calcium content began to decrease, and the PAL activity reached the maximum.

3.3.4 Changes in the water Content in the spathe of *A. andraeanum* cultivated with oyster shell

The regression analysis in Table 2 showed that the effect of T on the anthocyanin content in the spathe showed an increasing trend with the increase in treatment time. The results in Figure 6 show that increased time was associated with increased anthocyanin accumulation ($r=0.9829$), with a significant interaction detected between the anthocyanin content and treatment time ($F=28.5261^{*}$, $F_{0.05}=19.00$, $F>F_{0.05}$). At $T=3.1$ (w), the minimum anthocyanin content was 0.6723 ± 0.0342 (OD₅₃₀).

4 Discussion and conclusion

The solubility of oyster shells increased with the increase in fragments mass C and time T. The larger the fragments and the longer the time, the larger the solubility.

In oyster shell substrate, cultivation of *A. andraeanum* under low-light conditions (less than $220 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at pH 5.9, the PAL activity and anthocyanin content increased with time, and the soluble sugar content increased correspondingly. At 8 weeks, the activity of PAL reached its maximum, while at 15 weeks, the soluble sugar content reached its maximum, which resulted in a higher content of anthocyanin. This was similar to the conclusion of a previous^[5] study in which Ca^{2+} promoted PAL activity^[12].

Oyster shells have a natural multi-porous structure with adsorption and slow-release characteristics^[8] and can absorb N, P and other elements to stabilize the concentration of N and P in the rhizosphere environment of the substrate and provide sufficient nutrients for the synthesis of soluble sugar^[13,14]. When *A. andraeanum* was cultivated using 286 mg oyster shell fragments under low-light conditions at pH 5.9 for 10 weeks, the water content of the spathe reached a minimum value, the pedicel diameter (11 w) reached the maximum value, and the soluble sugar content (expected 15 w) reached the maximum value, thus overcoming the problem of photosynthate (mainly sugar) accumulation decline caused by insufficient light^[15]. At the same time, the anthocyanin content of the spathe also showed a rapid upward trend, showing a positive synchronization with flower quality indicators. The reason might be the soluble sugars provide abundant precursors for anthocyanin synthesis, thus increasing the content of anthocyanin.

Oyster shell dissolution is determined by environmental acidity. The lower the pH, the faster the dissolution and the greater the dissolution amount, and more Ca^{2+} is released. The PAL activity was closely related to Ca^{2+} content^[5], and its peak values were earlier than that of soluble sugar, but were indirectly related to anthocyanin synthesis, and anthocyanin content did not exhibit the maximum value at the end of the test period, indicating that the synthesis of anthocyanin was also affected by other factors^[16].

One of the main limiting factors for the decrease in the anthocyanin content in *A. andraeanum* is low-light intensity. In the soilless cultivation of *A. andraeanum* under low-light conditions (produced in tropical winter), using oyster shell fragments as exogenous calcium (and as the cultivation medium) could effectively improve the PAL activity of the pedicel and the anthocyanin content of the spathe, which not only alleviates the environmental pollution caused by abandoned oyster shells but also improves the planting benefit of *A. andraeanum*.

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