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Effect of Oyster Shell Supplementation to the Culture Medium on Anthocyanin Content in the Spathe of *Anthurium andraeanum* Lind.

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***Abstract. Anthurium andraeanum* Lind. is an economically important potted and fresh-cut flower species. However, reduced anthocyanin content under shaded cultivation impacts the color of the spathe, which has negative implications for the marketability of *A. andraeanum.* Thus, the present study evaluates the use of oyster shell supplementation to the cultivation medium for improving anthocyanin content. Appropriate calcium (Ca) can improve the activity of phenylalanine ammonia lyase (PAL), and PAL activity is positively correlated with anthocyanin content; nitrogen (N) and phosphorus (P) nutrients are closely related to anthocyanin synthesis. N and P nutrients and Ca can alleviate the color symptoms of *A. andraeanum* when anthocyanin content decreases under weak light (under 220 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Microdissolution of calcium carbonate, the main component of oyster shell, can provide better exogenous Ca and adsorb slow-release N and P. Selecting appropriate oyster shell fragments will be the key to *A. andraeanum* experimental cultivation under low light conditions. Using regression models and response surface methodology (RSM), the relationships between oyster shell fragments and anthocyanin content are promulgated. The main findings indicated that the Ca released from 286-mg oyster shell fragments at pH 5.5 significantly increased the activity of PAL in the pedicel under weak light within 8 hours. At pH 5.9, 375-mg oyster shell fragments could significantly adsorb N and P nutrients within 4 to 14 hours. In conclusion, 286 to 375 mg oyster shell fragments at pH 5.5 to 5.9 could stabilize slow-release fertilizer source and significantly increase anthocyanin content in *A. andraeanum* spathe.**

Anthurium andraeanum Lind. (Araceae) is an economically important tropical potted and fresh-cut ornamental flower species (Dufour and Guerin, 2003). Studies have shown that reduced light intensity under conditions of excessive shading or prolonged low temperature and rainfall in cultivation facilities leads to the fading of the spathe (Li et al., 2015; Wang et al., 2012), which seriously impacts the quality and marketability of *A. andraeanum* flowers (Chang et al., 2012).

A significant correlation was previously found to exist between Ca content in *A. andraeanum* and spathe discoloration, and exogenous Ca supplementation could significantly alleviate the symptoms of spathe discoloration (Henny and Hamilton, 1992; Higaki et al., 1980). Anthocyanins, as flavonoid pigments that contribute red, purple, and blue coloration to plants, are one of the key factors determining the color of the spathe of *Anthurium*. Research shows that PAL activity is positively correlated with anthocyanin content. Light can induce an increase in PAL activity (Xia and Cai, 2004), while Ca has been found to be positively associated with PAL and can thus produce the same effect as light. This finding implies that Ca can be used instead of light to stimulate increases in PAL activity, which might then result in the elevated production of anthocyanins.

Oyster shells are a solid waste byproduct of the mariculture industry and are composed of more than 90% calcium carbonate, thus providing a valuable Ca resource (Teng et al., 2019). Calcium carbonate can provide a good source of exogenous Ca in the soilless cultivation of *A. andraeanum* and can be used in the cultivation matrix. Oyster shells have a naturally porous surface and good solubility, adsorption, and chemical activity (Liu, 2004), and can adsorb ammonium nitrogen (NH_4^+–N). The use of oyster shell in the preparation of slow-release nitrogen fertilizer (Miao et al., 2007) can also alleviate the loss of N. The soilless cultivation of *A. andraeanum* using oyster shells depends on the size of the oyster shell fragments and the pH value of the solution. These two factors are closely associated with the dissolution of calcium carbonate and the absorption of N and P, and the factors may affect the quality of *A. andraeanum*.

The use of oyster shells for the supplementation of Ca in the cultivation medium of *A. andraeanum* has not been previously investigated. The present study thus aimed to test the use of soilless cultivation using oyster shell for increasing anthocyanin content in *A. andraeanum* by 1) determining the N and P absorption of differently sized oyster shell fragments, 2) evaluating the effect of different pH values on the release of Ca from the shells, 3) assessing the impact of temperature and light intensity on PAL activity and anthocyanin content, 4) evaluating the effects of oyster shell fragment mass and cultivation time on PAL activity in the pedicles, and 5) determining the changes in Ca^{2+} in the pedicles and anthocyanin content in the spathe under cultivation with oyster shells.

Materials and Methods

Materials

A 3-year-old red variety of *A. andraeanum* called 'Tropical' was grown in the Landscape Practice Teaching Base of Guangdong Ocean University (Zhanjiang, China). It was cultivated in a 5-L flow aquarium using a general nutrient solution for *A. andraeanum* supplemented with oyster shell fragments (one plant per box). Its new leaf sheath had been exposed for one week and was bronze in color. Five-centimeter-long flower branches were obtained, from which the pedicels were used for the determination of Ca and PAL, while the spathe was used for anthocyanin content determination. The oyster shells (collected from Zhanjiang market waste) were immersed in a 0.1% hydrochloric acid solution for 10 h. The surface sediment was cleaned using a wire brush, then the shells were dried naturally in a ventilated area. The oyster shells were crushed using a grinder and then screened.

Methods

Adsorption of the oyster shell fragments. The adsorption of total nitrogen (TN), ammonia nitrogen (NH_4^+–N), and total phosphorus (TP) by the differently sized oyster shell fragments in the general nutrient solution (Wang, 1997) was determined at different time periods. The optimum cultivation pH value for *A. andraeanum* was previously determined to be 5.5 to 6.5. Oyster shell fragments with masses of

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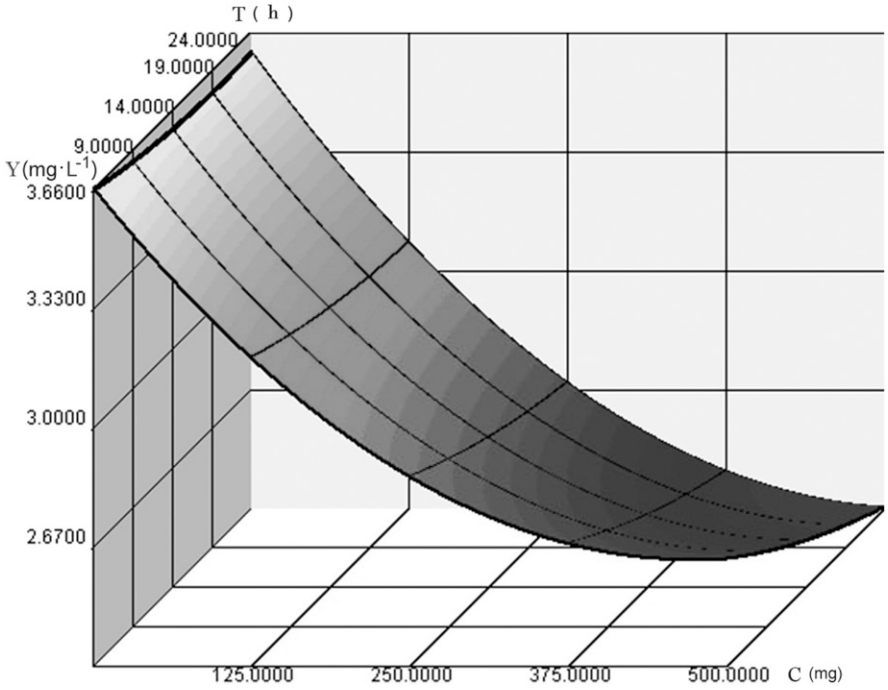

Fig. 1. Analysis of residual mass concentration of total nitrogen (TN) adsorbed by the oyster shell fragments by time (T) [$\hat{y}$ = TN = 3.708 – 0.003644C – 0.009343T – 0.0000207CT + 0.0000033C^2 + 0.00023T^2 (F = 44.7208**)]. Three-dimensional response surface plot showing the effect that when C = 720 mg and T = 52.6 h, the residual amount reached a minimum value of 2.1499 ± 0.2157 mg·L^{-1}.

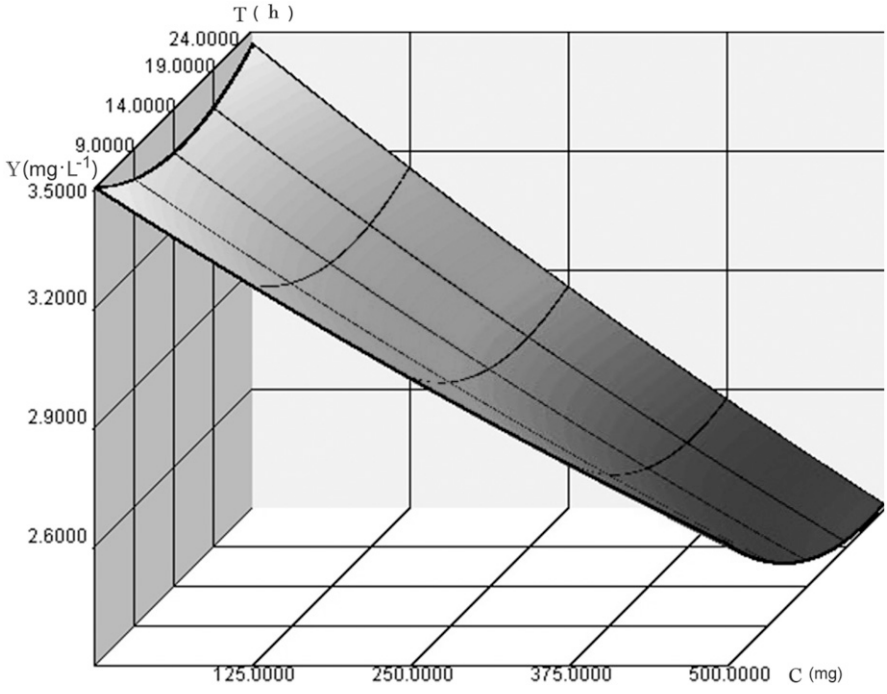

Fig. 2. Analysis of residual mass concentration of NH_4^+–N adsorbed by the oyster shell fragments by time (T) [$\hat{y}$ = NH_4+–N = 3.606 – 0.001597C – 0.02774T – 0.0000219CT + 0.000000362C^2 + 0.0009245T^2 (F = 11.5375**)]. Three-dimensional response surface plot showing the effect that when C = 4145 mg and T = 64 h, the residual amount reached a minimum value of –0.5943 ± 0.3435 mg·L^{-1}.

0, 125, 250, and 375 mg were placed in four 5 L flow aquariums at pH 6.0, and a sample solution was extracted at different time periods (4, 14, and 24 h). N and P adsorption on the differently sized oyster shell fragments were determined in 12 groups of flow aquariums and was repeated three times. The adsorption rate was calculated as $\eta = (C_0 - C_x)/C_0 \times 100\%$, where C_0 is the initial mass concentration for N or P in mg·L^{-1} and C_x is the residual mass concentration after adsorption.

Dissolution of the oyster shell fragments at different pH values and durations. The oyster shell fragments selected from the above test were soaked in 5-L phosphoric buffer (pH 5.7, 5.9, 6.1, 6.3, and 6.5) to test the dissolution at different pH values. The oyster shell fragments were removed regularly (every 1, 3, 5, 7, and 9 h), dried, and the residual weight measured. The dissolution of the oyster shell fragments under different pH values and durations was recorded. The pH was determined using an acidometer. The experiments were repeated three times.

Oyster shell supplementation on Ca^{2+}, PAL, and anthocyanins in A. andraeanum. The oyster shell fragments were combined with an *Anthurium* general nutrient solution (The nutrient solution was changed once a week.) The Ca^{2+} contents and PAL activity of the pedicels, and the anthocyanin content of the spathe of *A. andraeanum* treated at different treatment durations (4, 6, 8, 10, and 12 weeks) were determined under weak light (220 μmol·m^{-2}·s^{-1}; Xia and Cai, 2004). The treatments were repeated three times. Atomic absorption spectrometry was used for the extraction and determination of Ca content. The extraction and determination of PAL used the method of Ouyang G.C. (1985). Briefly, 0.2 g of pedicels from *A. andraeanum* was combined with borate buffer containing 5 mmol·L^{-1} rye-based ethanol, a small amount of polyvinylpyrrolidone (PVP), and sterile quartz sand. The mixture was ground in an ice bath and centrifuged at 12,000 rpm for 15 min, with the resulting supernatant constituting the crude enzyme extract. The supernatant was diluted four times with 1 mL; was absorbed at 0.8 mL; and was added to a 2 mL 0.1 mol·L^{-1} pH 8.8 borate buffer containing 2.0 mL 0.02 mol·L^{-1} L-phenylalanine, following which it was placed in a water bath at 30 °C for 1 h. The supernatant was measured at OD_{290} with an ultraviolet spectrophotometer. The determination of anthocyanin content followed the method of the East China Normal University's Biological Department (ECNU, 1980). Briefly, 0.2 g of spathe from *A. andraeanum* was weighed and cut into ≈2 to 3 mm fragments. The fragments were then placed into a plugged test tube. Twenty milliliters of 0.1 mol·L^{-1} HCl was added to the test tube, which was then placed in a constant temperature water bath at 32 °C for 4 h. The filtrate was filtered and measured spectrophotometrically at OD_{530}. The relative concentration unit of the anthocyanins was expressed 10 times.

Data analysis. The data were processed by Microsoft Excel 2013 and analyzed in SPSS Statistics 20.0 (IBM Corp., Armonk, NY). A nonlinear regression model with the quadratic function $y = a + bx + cx^2$ was used to analyze the results of each index in single factor and multilevel tests. Using the RSM, the following 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

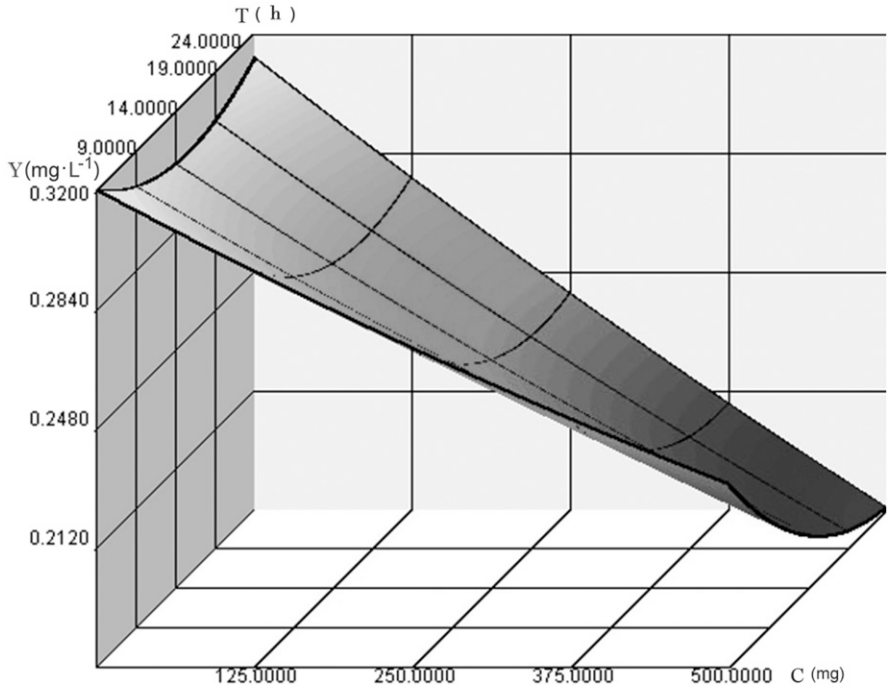

Fig. 3. Analysis of residual mass concentration of TP adsorbed by the oyster shell fragments by time (T) [$\hat{y}$ = TP = 0.3349 – 0.000155C – 0.003826T – 0.000004CT + 0.000000038C^2 + 0.000122T^2 (F = 5.2792*)]. Three-dimensional response surface plot showing the effect that when C = 20,738 mg and T = 354 h, the residual amount reached a minimum value of –1.9529 ± 0.0593 mg·L^{-1}.

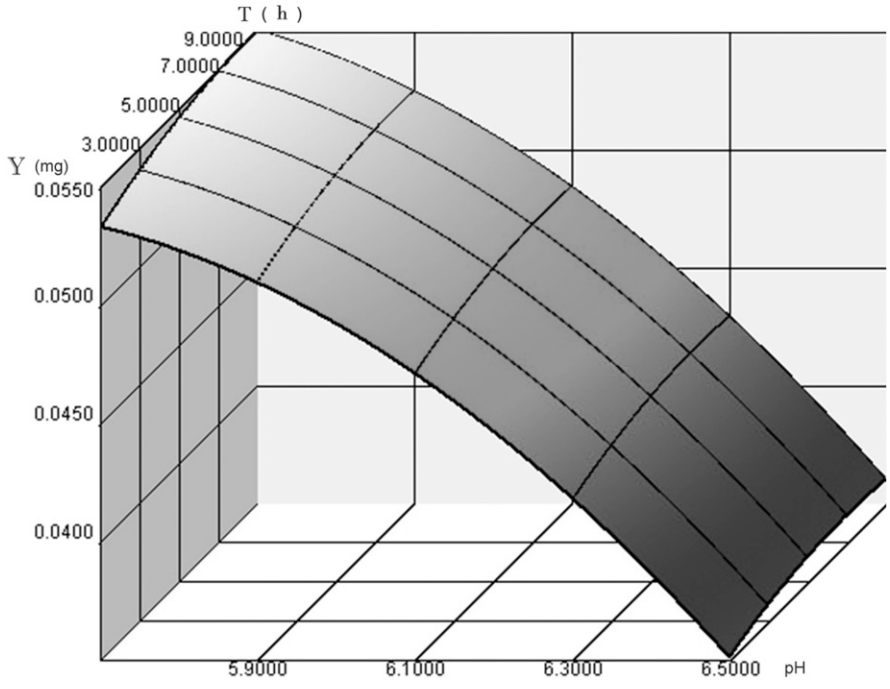

Fig. 4. Analysis of oyster shell fragments solubility (mg) under different pH values and time [T (h)] [$\hat{y}$ = dissolution = 0.503 + 0.2033pH + 0.001253T – 0.00125pHT – 0.01857pH^2 – 0.000032T^2 (F = 90.4048**)]. Three-dimensional response surface plot showing the effect that when pH = 5.4461, T = 8.916 h, the solubility reached a maximum value of 0.0564 ± 0.0032 mg.

multilevel test. The fit of the model was assessed using the coefficient of determination (R^2) and the adjusted R^2. The fitted polynomial equation was then illustrated using three-dimensional surface plots to visualize the relationships among the responses of the variables.

Results and Analysis

Analysis of the adsorption of N and P and the Ca released by dissolution from the oyster shell fragments

Analysis of the adsorption of N and P by the oyster shell fragments. The residual mass concentrations following adsorption are shown in Figs. 1–3. The adsorption of TN, NH_4^+–N, and TP from the oyster shells increased rapidly at first and then slowed down as time progressed. Within 4 h, the adsorption rate increased rapidly, slowing down after 14 h. One possible explanation is that the adsorption is mainly carried out on the surface of the oyster shell fragments or in the pores during the first 4 h, following which the increase in microporous ions in the oyster shell produces repulsion. This pattern would prevent the ions from approaching the oyster shell fragments in solution, thus slowing down the adsorption rate. The adsorption rates of TN, NH_4^+–N, and TP using 125-mg oyster shell fragments after 24 h were 55.6%, 39.1%, and 30.6%, respectively (TN > NH_4^+–N > TP), while these values were 57%, 49.5%, and 47.2% (TN > NH_4^+–N > TP), respectively, after 24 h using the 375 mg oyster shell fragments. The differences in adsorption rates were much more narrow in comparison with the 125-mg oyster shell fragments.

The adsorption of TN and NH_4^+–N differed significantly between the different oyster shell treatments ($F > F_{0.01}$ = 8.7459), whereas no significant difference in the adsorption of TP was observed ($F > F_{0.05}$ = 4.3874) (Figs. 1–3). The results indicate that adsorption increases with increases in oyster shell size, with the 375-mg oyster shell fragments exhibiting the best adsorption.

Effect of different pH values on the dissolution and release of Ca from the oyster shell fragments. The 375-mg oyster shell fragments were selected based on the above experiment, and the effects of different pH values (5.7, 5.9, 6.1, 6.3, and 6.5, respectively) on the dissolution and release of Ca from the shell were tested. The results are shown in Fig. 4. The dissolution of the oyster shell fragments was significantly correlated with pH and time (F = 90.4048**, $F_{0.01}$ = 4.1708, $F > F_{0.01}$). According to the RSM analysis (Fig. 4), the solubility of the oyster shell fragments increased with the increase in treatment time but decreased with the increase in pH value. The lower the pH value and the longer the treatment duration, the greater the solubility. At pH = 5.4461 and T = 8.916 h, the solubility of the oyster shell fragments peaked 0.0564 ± 0.0032 mg.

At the same time, the pH value of *Anthurium* soilless culture in actual production is between 5.5 and 6.5. Considering the adsorption effect of 375 mg and a 6.0 pH value of oyster shell fragments, pH = 5.9 was selected for the following experiments.

Effects of temperature and light intensity (PFD) on PAL activity and anthocyanin content in *A. andraeanum*

Comparative experiments of the effects of light and different temperatures on the growth of *A. andraeanum* showed that temperatures above 30 °C were too high for the plants, whereas temperatures below 15 °C resulted in yellow leaves, green back, and other harmful symptoms that significantly affected plant growth and development.

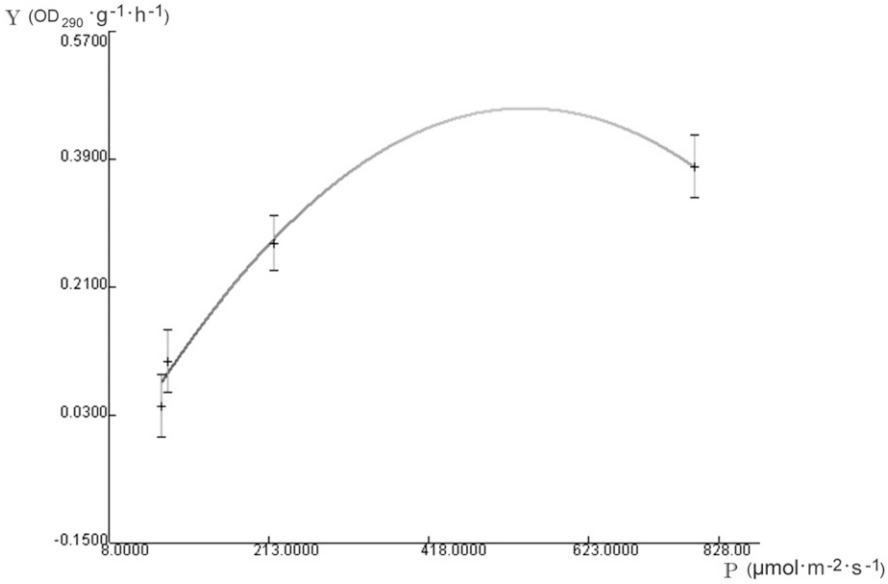

Fig. 5. Effects of PFD treatment with different light intensity on phenylalanine ammonia lyase (PAL) activity in pedicel [$\hat{y}$ = PAL = –0.0616 + 0.0016P – 0.000001757P^2 (F = 0.7981 < $F_{0.05}$)]. Two-dimensional response surface plot showing the effect that when P = 543, PAL activity has a maximum value of 0.4572 ± 0.1492 (OD_{290}·g^{-1}·h^{-1}) (r = 0.921, probability guarantee is 0.9081).

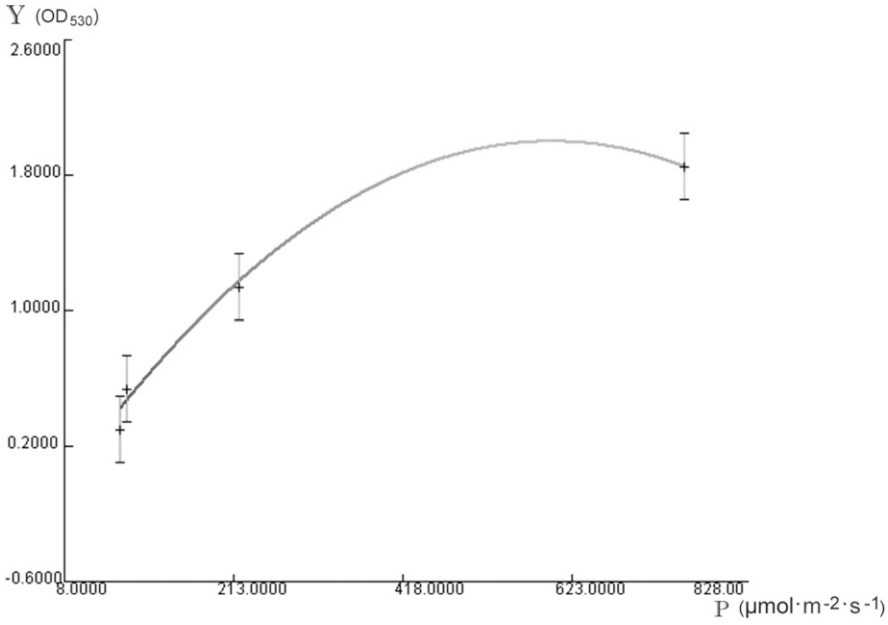

Fig. 6. Effects of PFD treatment with different light intensity on anthocyanin content in spathe [$\hat{y}$ = anth = –0.08235 + 0.0068P ± 0.00000568P^2 (F = 1.9024 < $F_{0.05}$)]. Two-dimensional response surface plot showing the effect that when P = 599, anthocyanin has a maximum value of 1.956 ± 0.8177 (OD_{530}) (r = 0.889; probability guarantee is 0.8267).

PAL activity and anthocyanin content in *A. andraeanum* under different light intensities at suitable cultivation temperatures (16 to 29 °C) confirmed that weak light intensity below 220 μmol·m^{-2}·s^{-1} could inhibit PAL activity and reduce anthocyanin content. Regression analysis showed that the PAL activity in the pedicels increased initially and then decreased with the increase in light intensity (Figs. 5 and 6). At P = 543 (P stands for light intensity, μmol·m^{-2}·s^{-1}), the PAL activity reached the maximum value 0.4572 ± 0.1492 (OD_{290}·g^{-1}·h^{-1}). While PAL activity and light intensity were not significantly associated (F = 2.7981, $F_{0.05}$ = 199.5, F < $F_{0.05}$), the obtained correlation r = 0.921 and determination R^2 = 0.9081 coefficients indicated a high correlation. Regression analysis (Fig. 6) found that the anthocyanin content of the spathe increased first and then decreased with the increase in light intensity. At P = 599 (μmol·m^{-2}·s^{-1}), the maximum anthocyanin content was 1.956 ± 0.8177 (OD_{530}). No significant association was observed between anthocyanin content and light intensity (F = 1.9024, $F_{0.05}$ = 199.5, F < $F_{0.05}$). The correlation r = 0.889 and determination 0.8267 coefficients were somewhat low, suggesting that light intensity may indirectly influence anthocyanin content.

Effects of oyster shell fragment mass *C* (mg) and cultivation time *T* (weeks) on PAL activity in the pedicles

The PAL activity in the pedicels cultivated under weak light (220 μmol·m^{-2}·s^{-1}) was determined by applying a general formula nutrient solution supplemented with shell fragments. The effects of C (mg) and T (weeks) treatment on the activity of PAL in the pedicels were studied. Regression analysis showed that the PAL activity in the pedicel first rose and then decreased with the increase in oyster shell fragment mass C (Fig. 7). When C = 286 (mg) and T = 8.1 (weeks), the activity of PAL reached the maximum value of 0.2936 ± 0.0838 (OD_{290}·g^{-1}·h^{-1}); when C exceeded 286 mg, the activity of PAL decreased with the increase in time. One possible explanation is that the increased duration resulted in a higher cumulative Ca content, which inhibited PAL activity and resulted in a decrease in PAL activity. There were significant differences in PAL activity between oyster shell fragment mass and time (F = 4.1232*, $F_{0.05}$ = 2.7401, $F > F_{0.05}$).

The results of the single factor test time for PAL activity showed that there was no significant interaction between PAL activity and the single factor of time (F = 0.7857, $F_{0.05}$ = 19.00, $F < F_{0.05}$); but when T = 7.4 (weeks), PAL activity had a maximum value of 0.2973 ± 0.0161 (OD_{290}·g^{-1}·h^{-1}). PAL activity was significantly affected by oyster shell fragment mass (F = 4.1232* > $F_{0.05}$), but not by time.

Content changes of Ca^{2+} in the pedicles and anthocyanin in the spathe under oyster shell cultivation

The contents of Ca^{2+} in the pedicels and anthocyanins in the spathe were determined under cultivation with 375 mg oyster shell fragments.

Ca^{2+} content in the pedicels increased first and then decreased with the increase in treatment time. Ca^{2+} content (mg) and treatment time (weeks) were not significantly associated (F = 1.694, $F_{0.05}$ = 19.00, F < $F_{0.05}$), indicating that the increase in Ca^{2+} content was not significant with time. However, at T = 8.5 (weeks), the Ca^{2+} content had a maximum value of 0.4900 ± 0.0482 (mg). It is possible that the accumulation of Ca^{2+} (with higher absolute value) inhibits PAL activity due to the increase of solubility of oyster shell piece. This characteristic can also be inferred from the correspondence of the peak values of Ca^{2+} and PAL (Figs. 8 and 9) occurring simultaneously in the eighth week.

The effect of T on anthocyanin content in the spathe showed an increasing trend with the increase in treatment time (Fig. 10). Increased time was associated with increased anthocyanin accumulation, with a significant interaction detected between anthocyanin

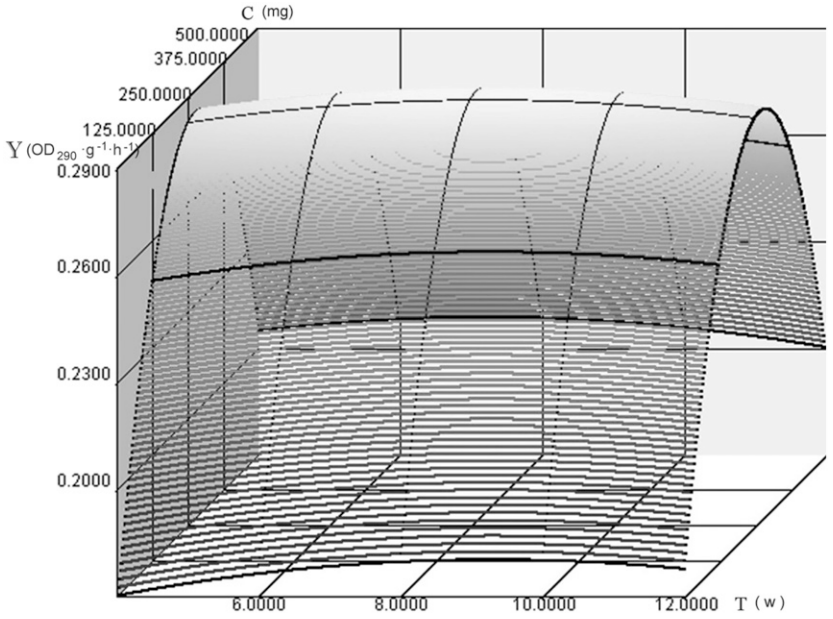

Fig. 7. Effects of oyster shell fragments' quality C (mg) and time [T (w)] treatment on phenylalanine ammonia lyase (PAL) ($OD_{290}\cdot g^{-1}\cdot h^{-1}$) activity of the pedicle in *Anthurium* [$\hat{y}$ = PAL = 0.1169 + 0.0068T + 0.001C – 0.0000033TC – 0.0003607T^2 – 0.000001772C^2 (F = 4.1232*)]. Three-dimensional response surface plot showing the effect that when T = 8.1438 w, C = 286.07 mg, then PAL activity has a maximum value of 0.2936 ± 0.0838 ($OD_{290}\cdot g^{-1}\cdot h^{-1}$). w = week

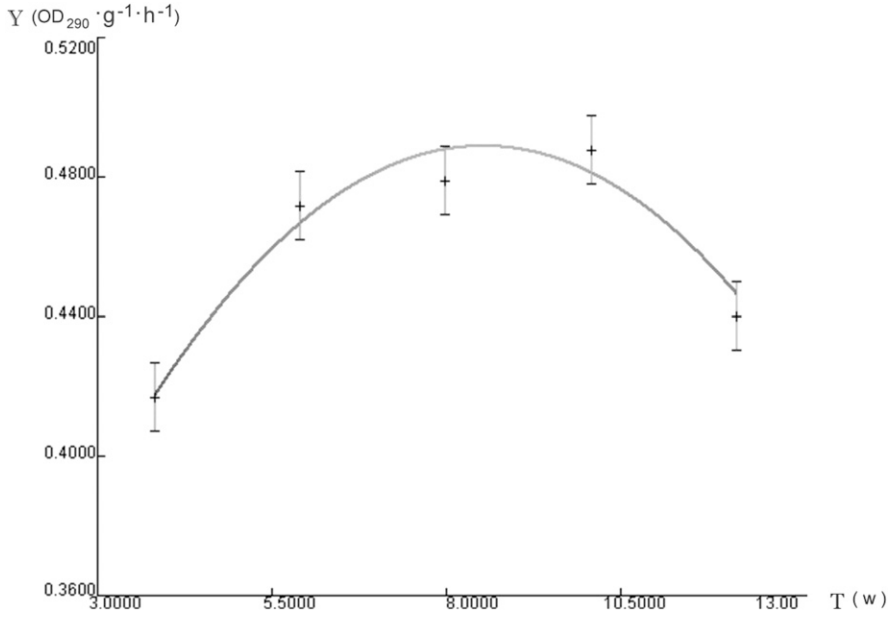

Fig. 8. Effects of time [T (w)] treatment on phenylalanine ammonia lyase (PAL) ($OD_{290}\cdot g^{-1}\cdot h^{-1}$) activity of the pedicle in *Anthurium* [$\hat{y}$ = PAL = 0.2562 + 0.0111T – 0.00075T^2 (F = 0.7857)]. Two-dimensional response surface plot showing the effect that when T = 7.4 w, PAL activity has a maximum value of 0.2973 ± 0.0161 ($OD_{290}\cdot g^{-1}\cdot h^{-1}$). w = week

content and treatment time (F = 28.5261*, $F_{0.05}$ = 19.00, $F > F_{0.05}$). At T = 3.1 (weeks), the minimum anthocyanin content was 0.6723 ± 0.0342 (OD_{530}).

Discussion and Conclusion

The results show that larger oyster shell fragments are associated with improved adsorption of N and P, with adsorption increasing rapidly initially and then slowing down with time. The oyster shell fragments with different masses at pH 5.9 value can significantly adsorb N and P within 4 to 14 h, exhibiting high adsorption rates high within 4 h, thus constituting a stable slow-release fertilizer source. We also discovered that lower pH values are associated with faster oyster shell dissolution. When the pH value of the solution was 5.5, maximum dissolution was achieved within 9 h. The microdissolution has a significant relationship with the pH value and time.

In the soilless culture of *A. andraeanum* using oyster shell fragments as a substrate, weak light intensity below 220 $\mu mol\cdot m^{-2}\cdot s^{-1}$ could inhibit PAL activity and reduce anthocyanin content. The effect of light intensity on PAL was higher than that on anthocyanins, indicating an indirect impact on anthocyanins (Gopaulchan et al., 2014; Xia and Cai, 2004). The Ca^{2+} released from the 286 mg oyster shell fragments at pH 5.5 significantly increased PAL activity in the pedicel under weak light within 8 h. In the 375 mg oyster shell fragment treatment at pH 5.9, the PAL activity of the pedicel was significantly increased under weak light. At 8 weeks, the Ca^{2+} content and PAL activity of the pedicel peaked, which significantly increased the anthocyanin content of the spathe, following which the anthocyanin content continued to increase. This finding suggests that the increase in anthocyanin content may depend on the increase of Ca^{2+} content and PAL activity over 8 weeks. Later, it is related to the slow release of N and P elements from oyster shell pieces to synthesize protein and sugar (Higaki et al., 1992).

$CaCl_2$ can effectively increase PAL activity and anthocyanin content in the pedicel (Avila-Rostant et al., 2010). It is proved that Ca^{2+} can overcome the decrease in photosynthate (mainly sugars) accumulation and PAL activity caused by insufficient sunlight (Wang, 1999; Wang et al., 2018) and can provide sufficient precursors and pathways for the synthesis of anthocyanins, thereby increasing anthocyanin content (Yang et al., 2015). The use of exogenous Ca^{2+} derived from oyster shell waste not only alleviates the environmental pollution caused by unused oyster shells but also offers a solution for improving the cultivation of *A. andraeanum*. In the future, it can also be used as a typical plant landscaping model under the tropical valley rainforest.

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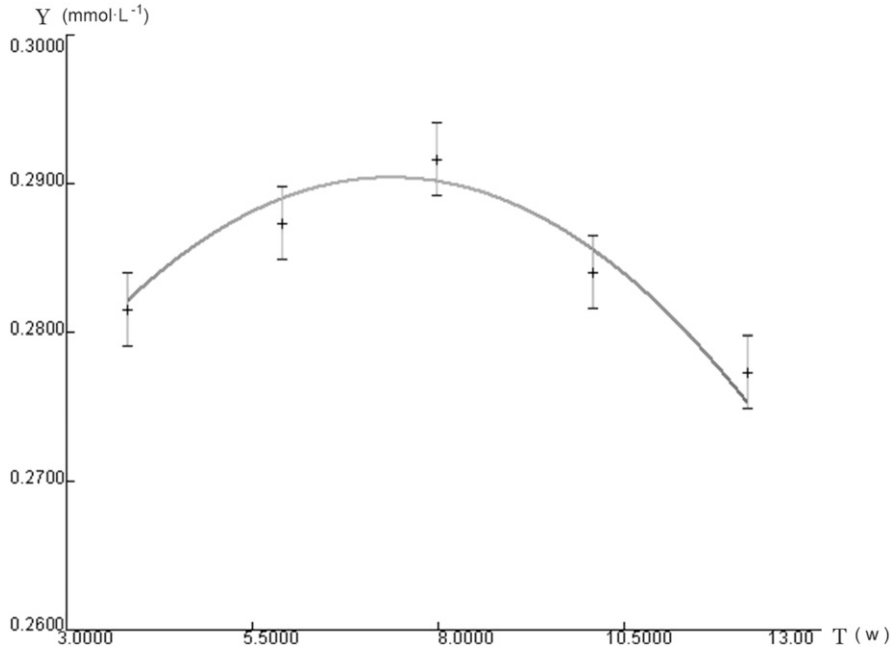

Fig. 9. Effects of time [T (w)] treatment on Ca^{2+} (mmol·L^{-1}) content in *Anthurium* pedicel [$\hat{y} = {}_{Ca2+} = 0.2439 + 0.05778T \pm 0.003392T^2$ ($F = 1.694$)]. Two-dimensional response surface plot showing the effect that when $T = 8.5158$ w, Ca^{2+} content has a maximum value of 0.4900 ± 0.0482 (mmol·L^{-1}). w = week

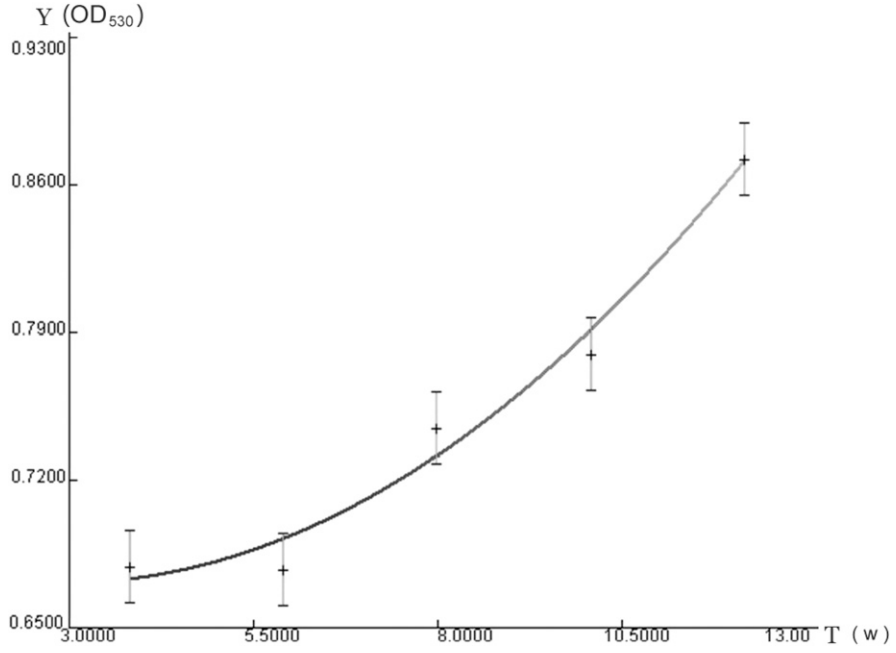

Fig. 10. Effects of time [T (w)] treatment on anthocyanin OD_{530} content in *Anthurium* spathe [$\hat{y} = {}_{anth} = 0.6967 \pm 0.01577T + 0.002535T^2$ ($F = 28.5261$**)]. Two-dimensional response surface plot showing the effect that when T = 3.1099 w, anthocyanin content has a minimum value of 0.6723 ± 0.0342 (OD_{530}). w = week

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