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Physiology Response and Resistance Evaluation of Twenty Coconut Germplasm Resources under Low Temperature Stress

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Abstract: Coconut (*Cocos nucifera* L.) is a tropical evergreen crop with high economic value. Low temperature is one of the main environmental factors that limit coconut productivity. Therefore, it is necessary and significant to research the growth trend and physiological changes of coconuts under a low temperature environment. In this study, the physiological response of 20 coconut germplasm resources is presented in an integrated perspective to provide a holistic view of the behavior of coconut trees facing cold stress under four temperature conditions (25 °C, 15 °C, 10 °C, 5 °C). It was shown that low temperature would lead to the increase of relative electrical conductivity, MDA content, soluble protein content, and proline content. In addition, the activities of defense enzymes (SOD, POD, CAT, APX) were increased to resist the cold environment. In a comprehensive analysis, it was revealed that coconut germplasms with high cold resistance, such as C2, C7, and C10 as well as POD activity, proline content, and soluble protein content, were defined as representatives for coconut cold resistance evaluation. Through the exploration of osmotic adjustment substances and defense enzymes, the breeding and quality improvement of cold-resistant coconut varieties could be promoted. As a result, understanding the physiological response and tolerance mechanisms of coconuts to low temperature stress was essential, as this perception may serve as the foundation for coconut resistance evaluation, cultivation, and breeding.

Keywords: *Cocos nucifera* L.; defense enzyme; low temperature; osmotic potential; physiological change

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1. Introduction

Coconut (*Cocos nucifera* L.), belonging to the *Palmae* family, is a tropical evergreen crop native to Southeast Asia and Pacific Islands Countries [1,2]. It is widely distributed in the tropical and subtropical regions of the world. Coconut palms are categorized as talls or dwarfs based on their size and stature. They are also monoecious. In other words, they are composed of male and female flowers on the same inflorescence (spadix) that grows within a woody spathe. The male and female flowers grow at various periods depending on the kind of coconut tree. Coconut is mainly seed-propagated, dwarf coconuts are autogamous, and the flowering is initiated after an average of 3 years; tall coconuts are allogamous and take longer to bloom [3]. Because coconut trees are propagated by seed, they are prone to numerous variances which may be seen in the trees, fruits, and leaves. Mass multiplication of elite coconut palms, with high yield and resistance to biotic and abiotic stresses, is the need of the hour for obvious reasons. Unfortunately, the progress achieved in clonal propagation in coconut has been rather sluggish. The recalcitrant nature of coconut is the main impediment for development of a commercial scale protocol for in vitro multiplication. Selection of explants is the key element for its successful outcome. Numerous tissues, i.e., leaves, inflorescence, plumular tissues, ovaries, anthers, roots, and zygotic embryos, have been utilized as explants for coconut tissue culture. Major advances

in the application of plant tissue culture techniques in coconut propagation are coconut embryo culture, immature embryo culture, and immature inflorescence culture. Coconut possesses extremely high edible and economic value among tropical fruit trees, for instance, coconut with rich nutrition can not only be tasted as fresh fruit, but also defined as a natural beverage. Additionally, mature coconut pulp is still an important raw material for oil extraction in production and processing, and processed products of coconut leaves, roots, stems, as well as coconut shells are also well received and favored [4]. Besides, coconut with high ornamental value [5] can also be widely used as street tree and garden landscaping, which becomes an iconic landscape plant in the tropics.

As an important economic plant in tropical regions, coconut production will be affected by a variety of environmental factors, which is also the main research direction of coconut in recent years. The physiological responses of coconuts under the influence of different abiotic stresses in several areas in Brazil were studied to investigate the relevant factors causing the phenomenon of low yield [6]. It was shown that the photosynthetic system of coconut seedlings in an arid environment would be irreversibly damaged, but the antioxidant enzyme activity was less affected under drought stress test [7], and the drought resistance physiological responses of different coconut seedling leaves caused by water stress have also been explored [8]. As a highly salt-tolerant plant, coconut osmotic substances were accumulated under salt stresses so that osmotic potential and water loss were reduced, which was also an important physiological response of coconut to environmental stresses [9]. In addition, there is a high temperature requirement for coconut cultivation; thus, temperature plays a very important role in the growth and production of coconuts [10,11]. The influence of high temperature stress on coconut pollination and seed setting rate were studied previously [10], and the finding of relevant physiological responses and substance content changes of coconut leaves under cold stress was beneficial for the markers of coconut resistance [12–14].

Low temperatures are a significant issue that impedes the natural growth of coconuts, imposing constraints on the development of the coconut industry [15]. Therefore, it is desperately in need of new varieties with better resistance to low temperature stress selection, breeding, and cultivation. Cultivating new cold-resistant germplasms, the adaptability and cold-tolerance of coconuts in low temperature can be enhanced, so that the production yield can be increased. However, the prerequisite for practice and application is markers in coconut cold resistance and corresponding physiological changes in coconut. There are few reports on the physiological research and cold tolerance evaluation of coconut under low temperature stress currently. Exploring the physiological responses of different coconut varieties under low temperature stress is of significance. Without doubt, the evaluation of plant cold tolerance cannot be judged solely by individual indicators [16]. It was comprehensive evaluation and comparison that can act in significant functions in physiological response and stress resistance markers [11], the cold resistance capabilities of diverse varieties are different [17], meaning that the investigation of cold response of several coconut germplasms is of great necessity.

In this study, the physiological responses of 20 coconut germplasm resources under low temperature stress were measured and explored. Multiple physiological indicators, including relative conductance, semi-lethal temperature, osmotic adjustment substance contents and defendant enzyme activities, were determined to investigate the physiological changes of coconuts. Furthermore, correlation analysis, cluster analysis, and principal component analysis were implemented for comprehensive evaluation and assessment. Germplasm resources are the basis of new varieties breeding and germplasm innovation [18,19], the identification and evaluation of germplasms can promote the in-depth development and utilization of resources [20]. The analysis and marker of cold resistance of coconut germplasm resources will lay the foundation for the selection and breeding of coconut and facilitate wider diversity of coconut resistant varieties that fulfills their current and future needs.

2. Materials and Methods

2.1. Plant Materials and Treatments

The plant materials used in this experiment were from the coconut resources nursery of Coconut Research Institute, Chinese Academy of Tropical Agricultural Sciences (19°33′14″ N, 110°47′14″ E, altitude 27.3 m). The uppermost, fully expanded leaves in each treatment were separated, and three-year-old coconut plants with strong, consistent growth and without disease were chosen as the sample crops. Twenty coconut germplasms were numbered as C1–C20, respectively, and the sources and types are shown in Table 1.

Table 1. Origins and types of experimental coconut materials.

Resource Number	Place of Origin	Type
C1	China	tall coconut
C2	China	tall coconut
C3	China	tall coconut
C4	China	tall coconut
C5	China	tall coconut
C6	Malaysia	dwarf coconut
C7	China	tall coconut
C8	China	dwarf coconut
C9	Vietnam	dwarf coconut
C10	China	dwarf coconut
C11	China	tall coconut
C12	Vietnam	dwarf coconut
C13	China	dwarf coconut
C14	China	tall coconut
C15	Malaysia	dwarf coconut
C16	Thailand	dwarf coconut
C17	Thailand	dwarf coconut
C18	China	tall coconut
C19	China	tall coconut
C20	Vietnam	dwarf coconut

In the light incubator, the potted coconut seedlings were placed with a relative humidity of 80–90%. Moreover, the light condition was white light at 5000–7000 lx with a photoperiod of 12 h. Four distinct temperature treatments were devised: 25 °C (CK), 15 °C (A), 10 °C (B), and 5 °C (C). After 24 h, the relative leaf conductivities were determined. Osmotic adjustment substance contents and defense enzyme activities of leaves were examined after 5 days of treatment. Every treatment was repeated three times and three plants were used in each repetition.

2.2. Assay of Relative Electrical Conductivity and Semi-Lethal Temperature

According to Dionisio-Sese and Tobita [21], relative electrical conductivity (REC) was determined and calculated. Fresh leaf tissue (0.1 g) was cut into 5 mm length and placed in a test tube containing 10 mL of distilled water. The test tube was sealed and heated with a water bath of 32 °C for 2 h, and the conductivity of the medium (EC1) was measured using an electrical conductivity meter (FE30, METTLER TOLEDO, Shanghai, China). Samples were autoclaved at 121 °C for 20 min to release all the electrolyte, and the electrical conductivity (EC2) was measured when cooled to room temperature.

The REC was calculated with the following formula:

$$REC = EC1/EC2 \times 100. \quad (1)$$

On the basis of the relative conductivities, the logistic function was fitted and semi-lethal temperature (LT_{50}) was calculated [22].

2.3. Determination of Osmotic Adjustment Substance Content

Malondialdehyde (MDA) content was determined with the thiobarbituric acid method [23] with a slight modification. Coconut leaf tissue samples (0.5 g) and 5 mL trichloroacetic acid (5 %) were thoroughly ground and centrifuged at 5000× *g*. The supernatant (2 mL) and 2 mL thiobarbituric acid (0.67 %) were mixed and heated in the water bath (100 °C) for 30 min. After cooling, the samples were centrifuged at 3000× *g* for 10 min. The absorbances of the supernatant at 450 nm, 532 nm and 600 nm were measured using a UV–VIS spectrophotometer (UV-1600, Aoyi, Shanghai, China). MDA concentration was calculated in accordance with the formula "$6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$" and MDA content was reported as μmol/g FW.

Referring to Bradford [24], soluble protein (SP) content was assayed. Fresh leaf samples (0.5 g) and 5 mL distilled water were ground thoroughly, and placed for extraction at room temperature for 30 min. The mixture was centrifuged at 4000 rpm for 20 min, then the supernatant was taken out and the volume was adjusted to 10 mL. Test solution (0.1 mL) and 5 mL Coomassie Brilliant Blue G-250 reagent containing ethanol and phosphoric acid were mixed and placed for 2 min. The absorbance value of the mixed solution at 595 nm was measured, and the SP content was expressed as mg/g FW.

Proline (Pro) content was determined according to Wang and Huang [23]. Fresh leaf tissue (0.5 g) and 5 mL sulfosalicylic acid (3%) were mixed up, and then extracted in a boiling water bath for 10 min with constant shaking during the process. After cooling, the extract (2 mL), 2 mL glacial acetic acid, and 2 mL ninhydrin including glacial acetic acid and phosphoric acid were mixed and boiled for 30 min to make the solution appear red. Toluene (4 mL) was added, and the solution was shaken for 30 s after cooling. After standing still for a moment, the supernatant was centrifuged at 3000 rpm for 5 min with a UV–VIS spectrophotometer (UV-1600, Aoyi, Shanghai, China). The absorbance of the mixture at 520 nm was determined, and Pro content was expressed as μg/g FW.

2.4. Determination of Defense Enzyme Activity

The SOD activity was assayed with assay kit (Nanjing Jiancheng Bioengineering Institute, China). Fresh leaf tissue samples (1.0 g) were ground into 10% homogenate with phosphate buffer (pH 7.0) and centrifuged at 4500 rpm for 10 min. The supernatant and related reagents were mixed fully and maintained with a water bath at 37 °C for 40 min. The chromogenic agent was added, and the absorbance was determined at 550 nm. One U was equivalent to the amount of enzyme corresponding to the 50% inhibition rate of enzyme in each milliliter of reaction solution.

According to the instruction of Nanjing Jiancheng Bioengineering Institute kit, the POD activity was determined. Leaf tissue samples (1.0 g) were ground in phosphate buffer (pH 7.0) and centrifuged at 4500 rpm for 20 min. The supernatant was mixed and reacted with the reagents and heated at 37 °C with a water bath for 30 min. The mixture was centrifuged at 4500 rpm for 10 min, and the absorbance of supernatant was assayed at 420 nm. One U indicated the quantity of enzyme that catalyzed 1 μg of substrate per minute.

The CAT activity was determined referring to the kit provided by Nanjing Jiancheng Bioengineering Institute. Leaf tissue (1 g) was mixed with phosphate buffer (pH 7.0) and centrifugated at 4500 rpm for 10 min. The crude enzyme supernatant was obtained and mixed with reagents for 1 min. The chromogenic reagent was added to measure the absorbance value at 405 nm. One CAT U was expressed as the amount that decomposed 1 micromole hydrogen peroxide per second.

Referring to Nanjing Jiancheng Bioengineering Institute kit, the APX activity was determined. The fresh leaf (1 g) samples were ground with the buffer in the kit and centrifuged at 10,000 rpm for 10 min. The supernatant, buffer solution, substrate solution, and matrix solution were mixed, and the absorbance value was measured at 290 nm in 10 s and 130 s. One U was represented as changes in the amount that catalyzed one micromole of ascorbic acid per minute in per milliliter of the system.

2.5. Data and Statistical Analysis

All measurements were repeated three times in this experiment. The data were presented as the mean ± standard deviation (SD) (n = 3). The differences between treatments were analyzed by one-way ANOVA, and the significant differences among means were identified by Duncan's test at probability levels of 0.05 and 0.01 using SPSS software (version 19.0, IBM). Correlation analysis, principal component analysis, and cluster analysis were carried out using Data Processing System (DPS, version 9.01).

3. Results and Discussion

3.1. REC and LT_{50}

The structure and function of cell membranes produce important influences on the adaptability of crops to adversity [25]. The material exchange between plant cells and the external environment is carried out through biofilms, and the effects of various adversities and damages on plant cells also perform on biofilms first [26]. REC, a reflection of the condition of plant membrane system, is an important physiological indicator for identifying the permeability and damage of the cell membrane [27], which can be defined as a significant factor for crop resistance through breeding and cultivation. The relative conductivities of different coconut germplasm resources at different low temperatures were fluctuated, as shown in Figure 1, and the relative leaf conductivity was higher when temperature was lower. The values of all varieties at 25 °C were significantly different from other three temperature treatments, most of the germplasms showed obvious differences under different temperature treatments, indicating that low temperature stress acted in relative electrolyte leakage changes of coconut leaves. Perhaps the low temperature caused damage to plant cell membrane, leading to the increase of membrane permeability and the extravasation of intracellular substances, enabling relative conductivity to increase as temperature declined [28]. We speculate similar findings under low temperature stress in strawberry as recorded by Cheng et al. [29]. Among the 20 coconut leaves tested, C4, C12, and C19 manifested lower values at 5 °C (Figure 1), illustrating that these germplasms possessed strong abilities to resist cold condition, which could be considered important for cold-resistant cultivars. The cells of C6 and C7 were severely damaged under cold stress, indicating that these two resources could not be utilized as cold resistant breeding and cultivation materials. Additionally, varieties with numbers of C11, C12, C15, C16, and C17 exhibited low relative conductivities at room temperature, which might indicate that the afore mentioned varieties mesophyll cells were less damaged at normal temperature, resulting in high field quality and condition. It could be seen that C5 manifested highest value at 25 °C and lower at 5 °C, showing the lowest increase (2.515 times than control) in relative conductivity, while C11 appeared the highest increase, reaching 18.44 times that of the control group. From another perspective, C5 had a better cold tolerance under low temperature stress but C11 was not among the cold resistant varieties.

LT_{50} is widely used to reflect the limit of crop response to low temperature [30–32]. As an important indicator to measure cold tolerance of plants, the semi-lethal temperature can reflect the cold tolerance and low temperature limit of crops directly and accurately, which is significant for introduction and domestication of plants [33]. The semi-lethal temperatures obtained from the REC combining the Logistic function of several germplasm resources was shown in Figure 2. It could be seen that C5 manifested the lowest value (0.33 °C), while C20 presented the highest (2.03 °C), which was 6.15 times that of C5. Furthermore, the LT_{50} of C2, C3, C4, and C14 were also relatively low, indicating that these germplasms might possess a low tolerance limit to low temperature. From this perspective, the cold resistance ability of C20 was poor, and it could not be recognized as a good cold-resistant germplasm.

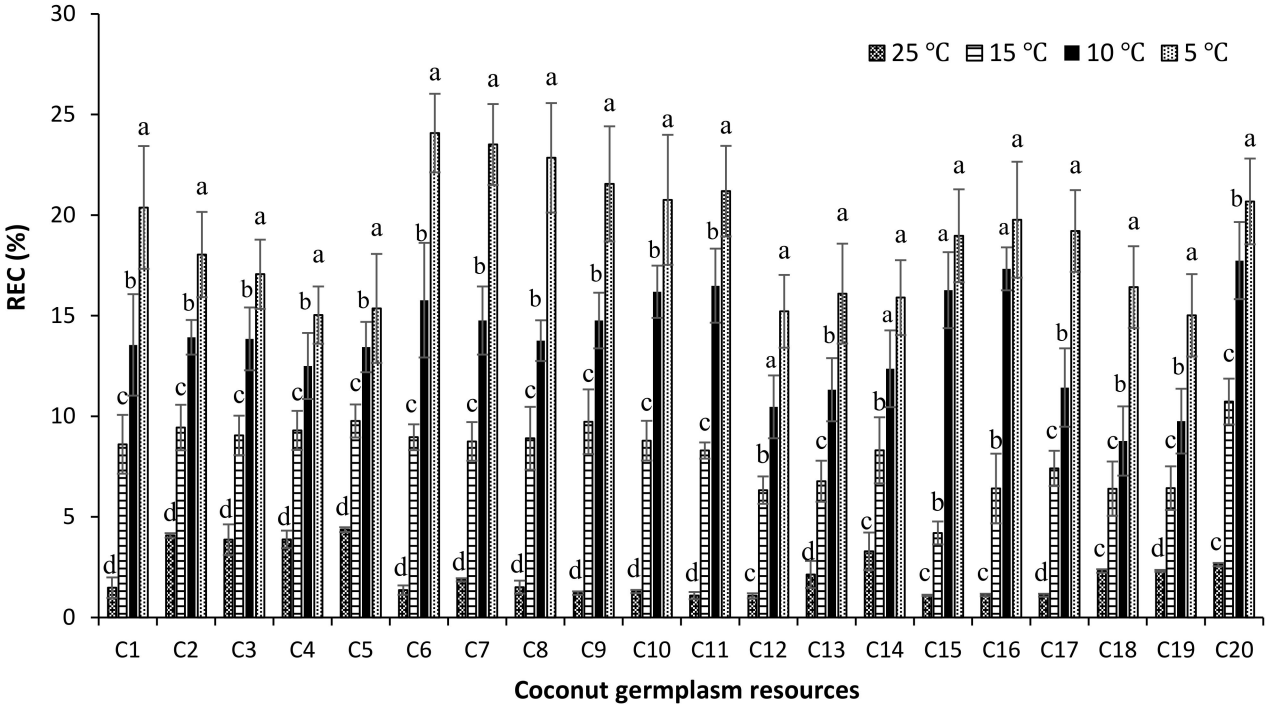

Figure 1. Changes of REC of 20 coconut germplasm resources under different temperature treatments. The data are presented as the mean ± standard deviation (SD). Different lowercase letters indicated significant difference ($p < 0.05$).

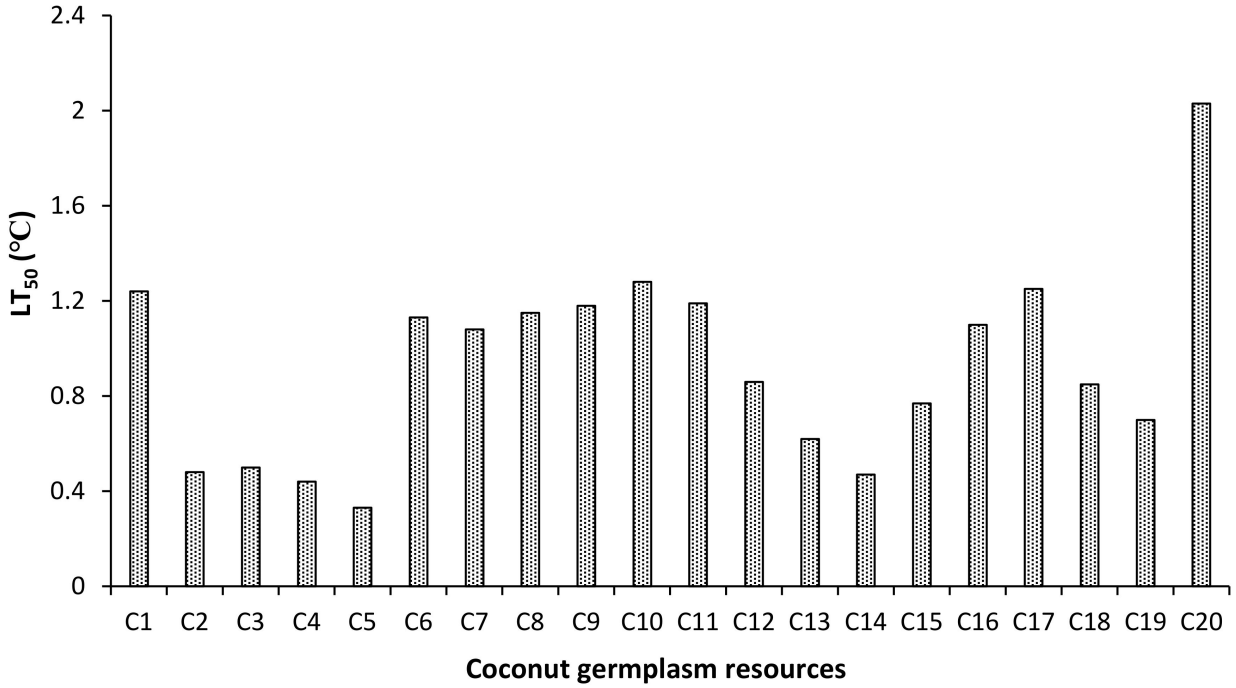

Figure 2. Changes of LT_{50} of 20 coconut germplasm resources.

3.2. Osmotic Adjustment Substance Content

Plants may be induced to undergo osmotic stress under low temperature stress and the cell membrane structure will be changed or degraded, resulting in leakage of solute out of the cell. In order to maintain stable osmotic pressure in plant cell, plants will strive to reduce the low temperature damage by adjusting the contents of osmotic adjustment substances [34,35].

3.2.1. MDA Content

As a product of membrane lipid peroxidation, MDA can reflect the structural integrity of biological membranes [36], and it is widely used as an important physiological index for the evaluation of cold tolerance of plants [37]. As shown in Figure 3, the MDA content of the tested coconut leaves increased gradually as the temperature decreased. While comparing the four temperature treatments, it could be found that MDA content in the leaves increased slowly under the environment of 25 °C to 10 °C, indicating that these temperature conditions had minimal effect on the cold damage of coconuts. The content of C11 under 10 °C reached the highest value, which increased by 1.80 times compared with the 15 °C treatment, and MDA content decreased at 5 °C. It might be speculated that C11 was more sensitive to 10 °C environment, and the membrane lipid peroxidation reaction was violent, resulting in a large amount of ROS production, so as to damage the biofilm and accumulate a large amount of MDA [38]. MDA content of other coconut germplasms was highest at 5 °C, which was significantly higher than other temperature treatments. The discrepancies in MDA peak values between C11 and others could be attributable to the molecular biological differences, which need further discussion. C5 manifested the lowest MDA contents and growth rates at 5 °C, indicating that it had less membrane lipid damage under low temperature stress and possessed higher cold tolerance. By contrast, C2, C8, and C9 presented higher contents and growth rates, which were susceptible to cold damage. Moreover, MDA contents were reduced in C1, C6, and C13 at 5 °C, but the growth rates were higher than 3.7 times, indicating that low temperature (5 °C) conditions had a great impact on the infiltration system. It was also pointed out that the plant cold tolerance could not be evaluated only by measuring the single indicator, and it was necessary to combine with other indicators such as REC and other osmotic adjustment substances for comprehensive analysis [38]. In general, low temperature stress would significantly accelerate the production of ROS in coconuts and enhance the oxidative stress response of plant cells. The increase of ROS destroyed the membrane lipid structure and affected the function and integrity of biofilms, so as to lead to excessive accumulation of MDA [27,39,40], which was consistent with previous study [26,41,42].

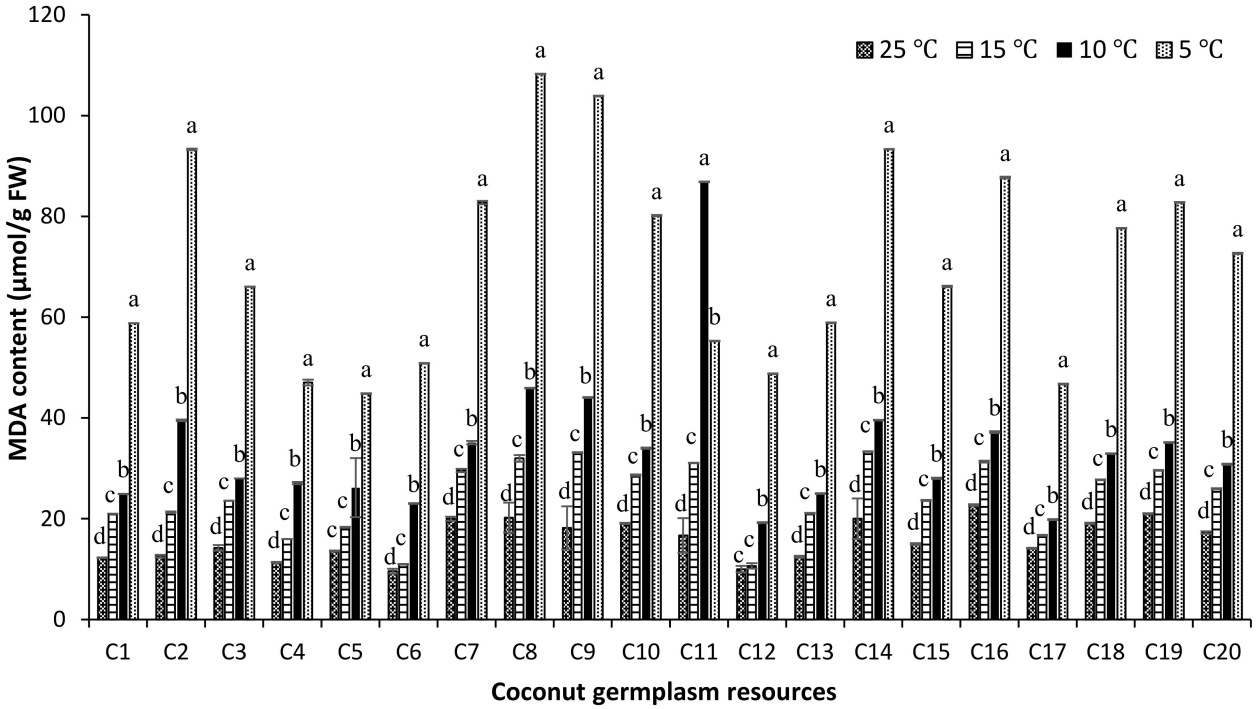

Figure 3. Effects of different low temperature treatments on MDA contents of 20 coconut germplasm resources. The data are presented as the mean ± standard deviation (SD). Different lowercase letters indicated significant difference ($p < 0.05$).

3.2.2. SP Content

The response of plants to low temperature is a complex process in which protein plays an important role in maintaining the homeostasis of ions in cells [43]. Furthermore, SP, which possesses good hydrophilicity at low temperature, can reduce intracellular water loss, indicating that the SP content in plant cells has a close relationship with membrane lipid penetration, and therefore SP plays a quantitative and qualitative role in crop cold tolerance [44]. In this study, SP content in leaves of coconut germplasm resources increased first and then decreased with the decrease of temperature, reaching the highest value at 10 °C, and the content differed significantly between different temperatures (Figure 4). Among the cultivars, C16 manifested the highest content of 105.97 mg/g FW at 10 °C, and C7, C15, and C20 also presented relatively high values. At the same time, the growth rates of SP content in these resources with the decrease of temperature were also larger, illustrating that their osmoregulation was stronger when exposed to low temperature. On the contrary, the content and growth rate of leaves of C1, C2, and C3 were the lowest. Proteins can react physiologically and biochemically with lipids on biological membranes to reduce membrane permeability, thereby regulating the osmotic function of plants and enhancing the cold resistance [45]. It was shown that as the temperature dropped below 10 °C, the variants C7, C15, and C20 manifested better cold resistance, while C1, C2, and C3 varieties exhibited less resistance. The SP content in the leaves dropped at 5 °C which might be related to significant cold damage to coconuts at this point, which exceeded the tolerance range, causing an imbalance of intracellular osmosis and a decrease in protein content. The increase of SP content in coconuts under low temperature stress reflected the physiological response to cold, which was consistent with previous studies [46,47]. In addition, protein in plant cells acted as a protective factor when exposed to low temperature [48]. In a cold environment, some specific proteins were synthesized in large abundance, which may function in the cell sap to inhibit the accumulation of ice crystals, thereby reducing the damage to plants caused by chilling injury [49].

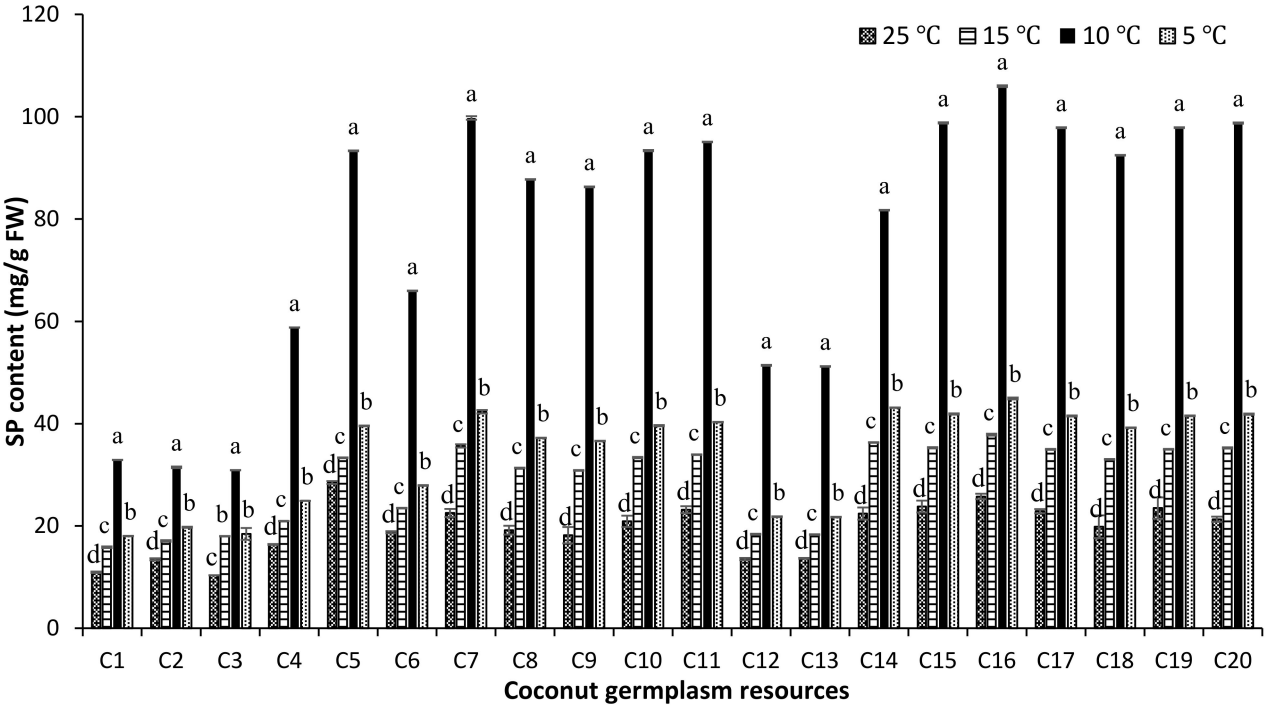

Figure 4. Effects of different low temperature treatments on SP contents of 20 coconut germplasm resources. The data are presented as the mean ± standard deviation (SD). Different lowercase letters indicated significant difference (p <0.05).

3.2.3. Pro Content

Pro is widely used in assessing plant resistance to diverse abiotic stimuli, because it helps to regulate and maintain cell osmotic balance and plays an important role in reducing cell redox potential reactions [50,51]. Meanwhile, Pro functions as a highly potent non-enzymatic antioxidant, participating in the antioxidant response of plant cells [52]. It was ascertained that the Pro content in the leaves of different coconut germplasms first increased and subsequently reduced when the temperature declined (Figure 5). Pro content peaked at 10 °C and showed significant differences between temperature treatments. C8, C10, and C11 showed higher contents at a low temperature of 10 °C, which was 2.02 times that of the control. Moreover, the content of C4 was the lowest (only 46.34 μg/g FW) among the 20 coconut germplasms, but it possessed a higher growth rate of Pro content. Under the stimulation of low temperature stress, the production rate of ROS in coconut leaves was accelerated, membrane lipid peroxidation was enhanced, and cell membrane lipids were damaged, resulting in leaves accumulating significant quantities of proline [53]. As an antioxidant and a free radical scavenger, Pro can scavenge active oxygen, protect protein, and cell membrane structure [54]. Pro, like soluble protein, has a high solubility that allows it to lower the freezing point of plant cells, inhibit water loss, and maintain cell osmotic balance, which together enable plants to improve the stress resistance [55,56]. The decrease in Pro content at 5 °C might be influenced by the fact that the temperature was too low to cause serious damage to the cell membrane, and the regulation and protection mechanism of proline was destroyed, resulting in the loss of intracellular material and the decrease of Pro content [57].Therefore, increasing Pro content at low temperature was conducive to improving the tolerance of plants, and Pro content could be defined as an important indicator to evaluate plant resistance.

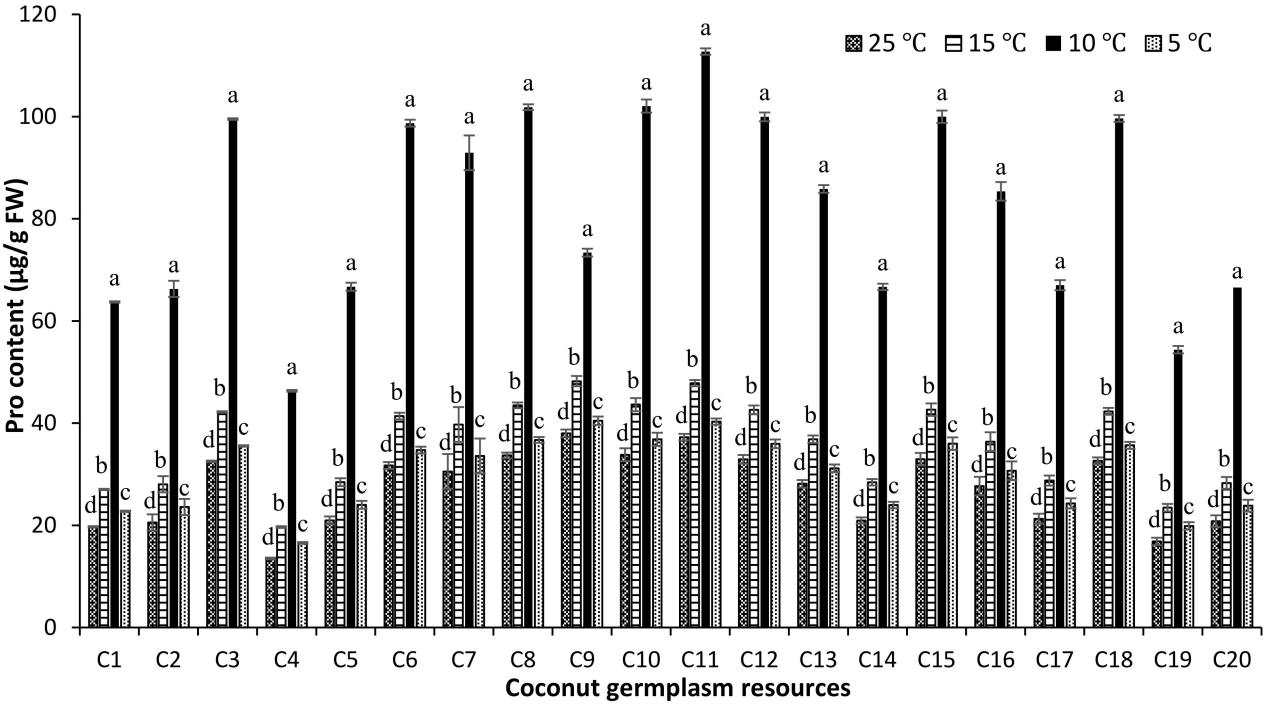

Figure 5. Effects of different low temperature treatments on SP contents of 20 coconut germplasm resources. The data are presented as the mean ± standard deviation (SD). Different lowercase letters indicated significant difference ($p < 0.05$).

3.3. Defense Enzyme Activity

Reactive oxygen species (ROS) are continuously produced and accumulated in the life process of plants, leading to oxidative stress and toxicity [58], causing oxidative damage to plant cells and inhibiting the growth and development of crops. The response of plants to environmental stress is closely related to ROS and the related protective enzyme

system [59]. In general, the ability to scavenge reactive oxygen species can be increased by upregulating enzyme activity, thereby resisting adverse environments [60–62]. It was clear that the synergistic reactions of SOD-POD-CAT-APX systems were effective against oxidative damage in plants to abiotic stress [27,63]. In this study, the activities of SOD, POD, and CAT in all tested coconuts showed a trend of gradually increasing with the decrease of temperature (Figures 6–8), indicating that the cold stress activated defense enzyme system to resist the low temperature, which was consistent with previous researches [64,65]. In terms of APX activity, the tested varieties displayed a tendency to increase first and then decrease, reaching highest at 10 °C, which was similar to Cao et al. [11]. In most coconut germplasms, all enzyme activity showed substantial variations across four temperature treatments, indicating that the four enzymes in coconuts were relatively sensitive to low temperature.

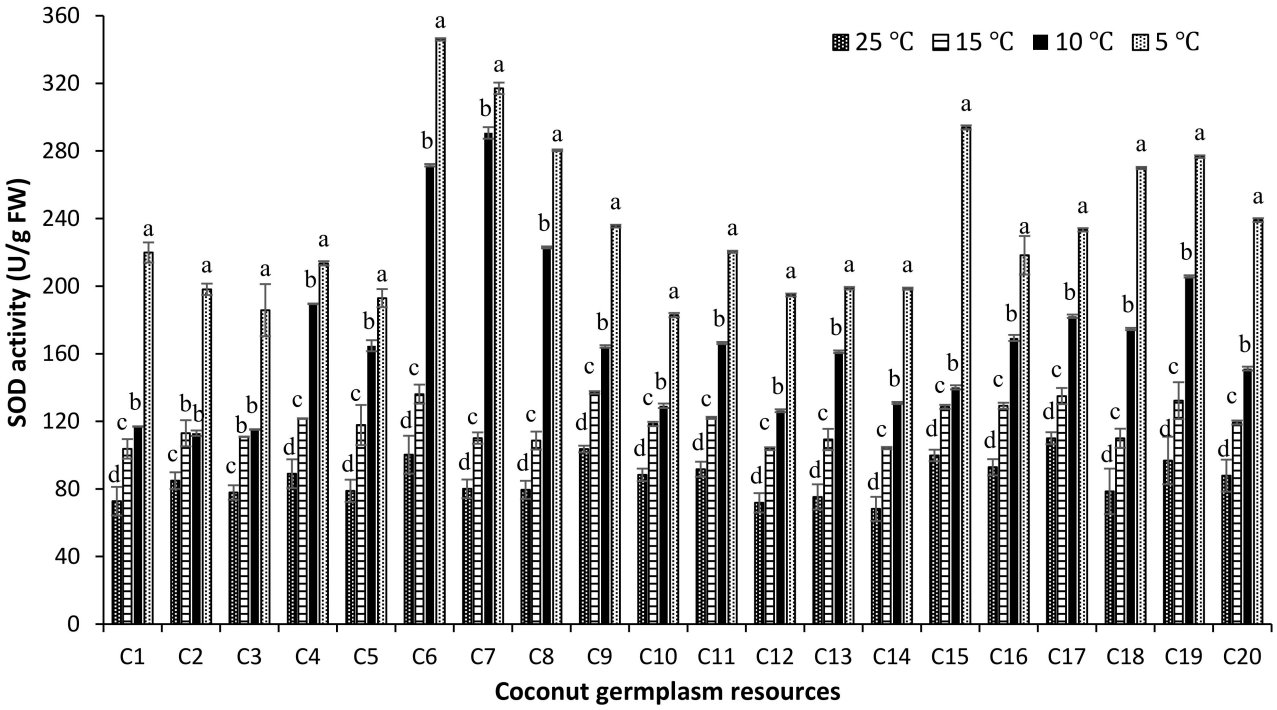

Figure 6. Effects of different low temperature treatments on SOD activities of 20 coconut germplasm resources. The data are presented as the mean ± standard deviation (SD). Different lowercase letters indicated significant difference ($p < 0.05$).

The coconut resources C6, C7, and C15 emerged with higher SOD activity at 5 °C, while C10 presented the lowest (182.86 ± 1.26 U/g FW), which was nearly half of C6 (Figure 6). At room temperature, C6, C9, and C17 had higher values, indicating that these varieties presented less cell damage. It was interesting that SOD activity of C2 and C3 at 15 °C and 10 °C were similar, while C6, C7, and C8 showed significant differences at two times ($p < 0.05$), reflecting the diversity of cold responses of several coconut varieties. Although the changes of POD activities were gradual, and the range of the value change was not so large, nevertheless, activities of different temperature treatments appeared to differ significantly (Figure 7). When compared to other resources, C2, C6, C12, and C14 exhibited high POD activities at 5 °C, implying a stronger ability to resist the 5 °C environment; while C1, C2, and C16 presented higher values at 10 °C, which indicated that the tolerance of various germplasms to low temperature at different temperatures was also different. Thus, it was necessary to investigate the cold resistance of several coconut resources and explore the suitable cultivated germplasms at corresponding temperatures. The antioxidant defense system includes a series of components that respond to oxidative stress and protect cells. As the first line of the defense system [66], SOD can promote the conversion of harmful free radicals and reduce cell damage [67–69]. POD can prevent the formation of

reactive oxygen species and the formation of free radicals, so that cell membrane damage can be reduced [70,71]. Furthermore, POD, a significant enzyme involved in the antioxidant system [72], can promote the synthesis of lignin when decomposing hydrogen peroxide, which is conducive to the lignification of cell walls, thus help plants resist the adverse environments [73,74]. Higher SOD and POD activities could scavenge reactive oxygen species to improve the cold tolerance of plants; consequently, C6 and C7 with higher values could be characterized as cold resistance cultivars.

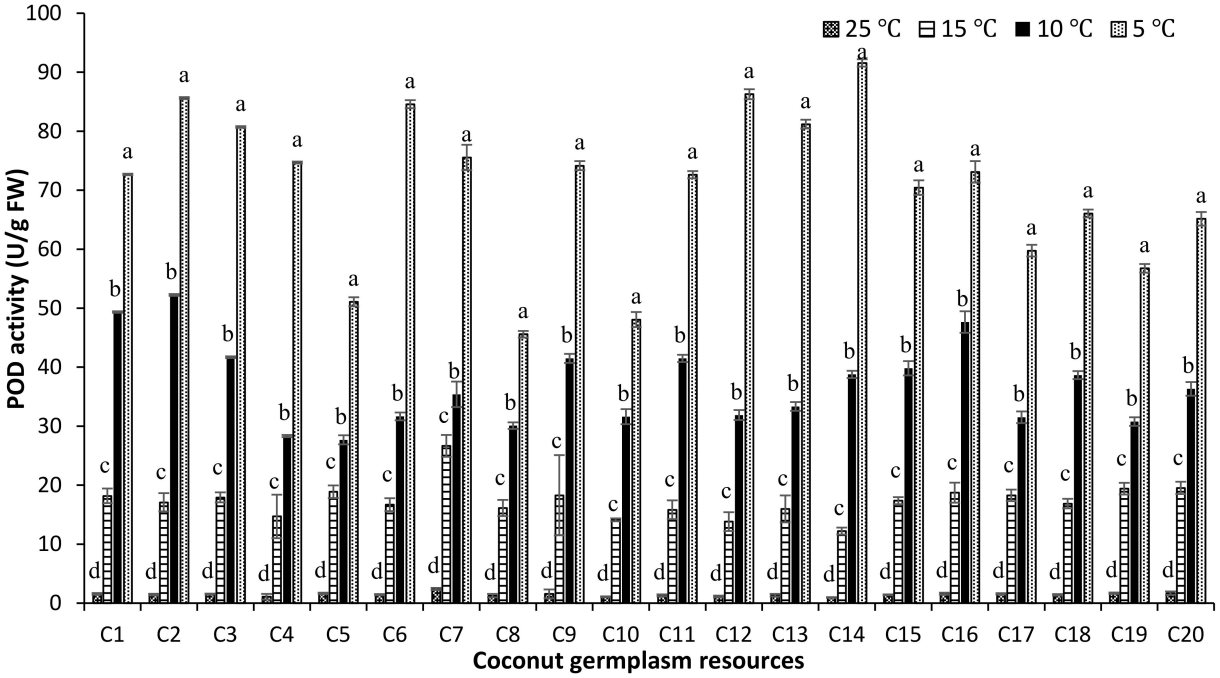

Figure 7. Effects of different low temperature treatments on POD activities of 20 coconut germplasm resources. The data are presented as the mean ± standard deviation (SD). Different lowercase letters indicated significant difference ($p < 0.05$).

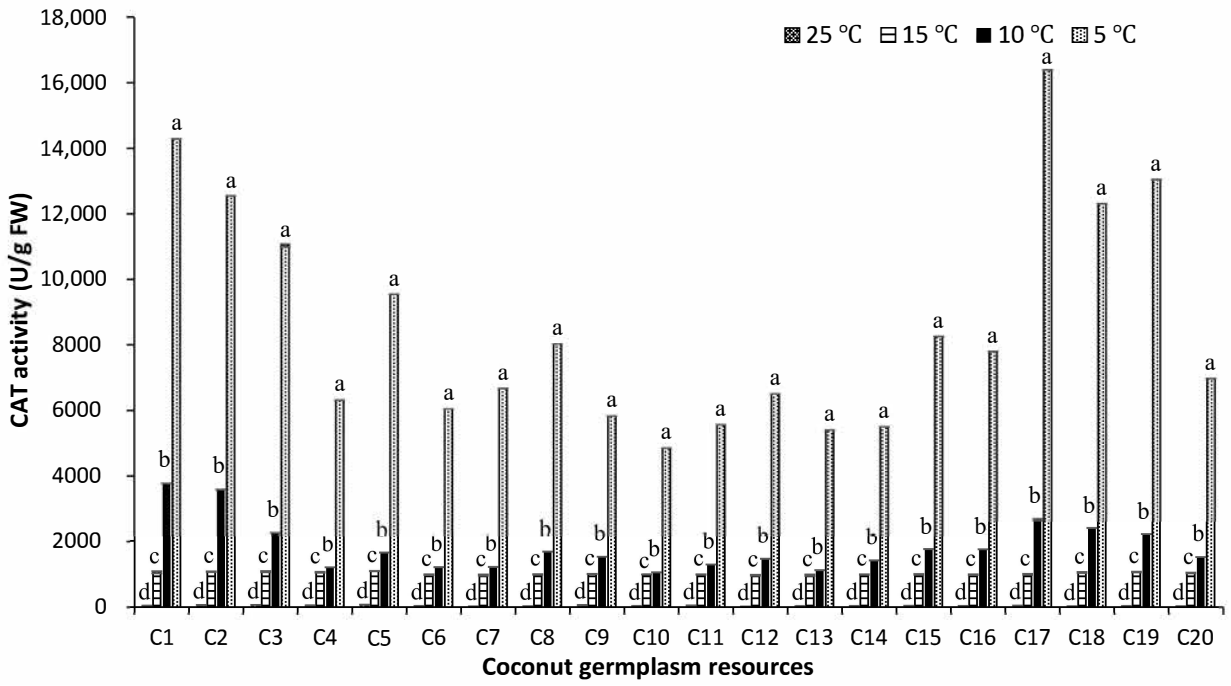

Figure 8. Effects of different low temperature treatments on CAT activities of 20 coconut germplasm resources. The data are presented as the mean ± standard deviation (SD). Different lowercase letters indicated significant difference ($p < 0.05$).

The changes of CAT activity with temperature presented were most dramatic (Figure 8). C17 and C1 manifested higher CAT activities at 5 °C, showing 366 times and 324 times than control, respectively. Furthermore, the two varieties showed higher values at 10 °C, indicating they possessed higher cold resistance and might be the superior for cold hardiness cultivation. It could be seen that the activities of all coconuts at 15 °C were relatively close, which might indicate that most of the tested varieties had minimal variation in tolerance to 15 °C. The CAT activities of C11, C13, and C14 were low, illustrating that they were not fit for cultivation in cold environments from a CAT perspective. Unlike the other three enzymes, APX activities in most germplasm resources increased and eventually decreased (Figure 9), which was similar to Jia et al. [75]. C7, C11, and C12 showed increased APX activities in three treatments (15 °C, 10 °C, and 5 °C), which indicated that these three might possess stronger ability to lower temperature (5–15 °C). Moreover, C2 and C19 presented the lowest values at 5 °C, which was contradictory to the high POD activity of C2, meaning that the markers of cold resistance needed to be completed comprehensively through multiple indicators. As a free radical scavenging factor [76,77], CAT can decompose hydrogen peroxide into water and oxygen [78] to maintain intracellular oxidative balance, reduce cell damage and improve the crop resistance [79,80]. Furthermore, APX is a component of the ascorbate-glutathione pathway, which plays an important role in balancing ROS and reducing oxidative damage to protect crops from stresses [81,82]. Higher CAT and APX activities could be due to the stronger cold resistance of coconut germplasm resources. As functioning in scavenging hydrogen peroxide during stress conditions, CAT, a predominant peroxisomal enzyme, exists in the peroxysome and mitochondria [83]; besides, APX mainly presents in chloroplast. CAT can catalyze breakdown of hydrogen peroxide with no external utilization; APX plays a key role in promoting the conversion of hydrogen peroxide into water using ascorbate as a specific electron donor [84,85], and APX possesses a higher affinity for H_2O_2 [86]. The different activity trends of CAT and APX might be due to different hydrogen peroxide contents generated by the cold stress in coconut cells, so that CAT and APX in diverse localizations were activated.

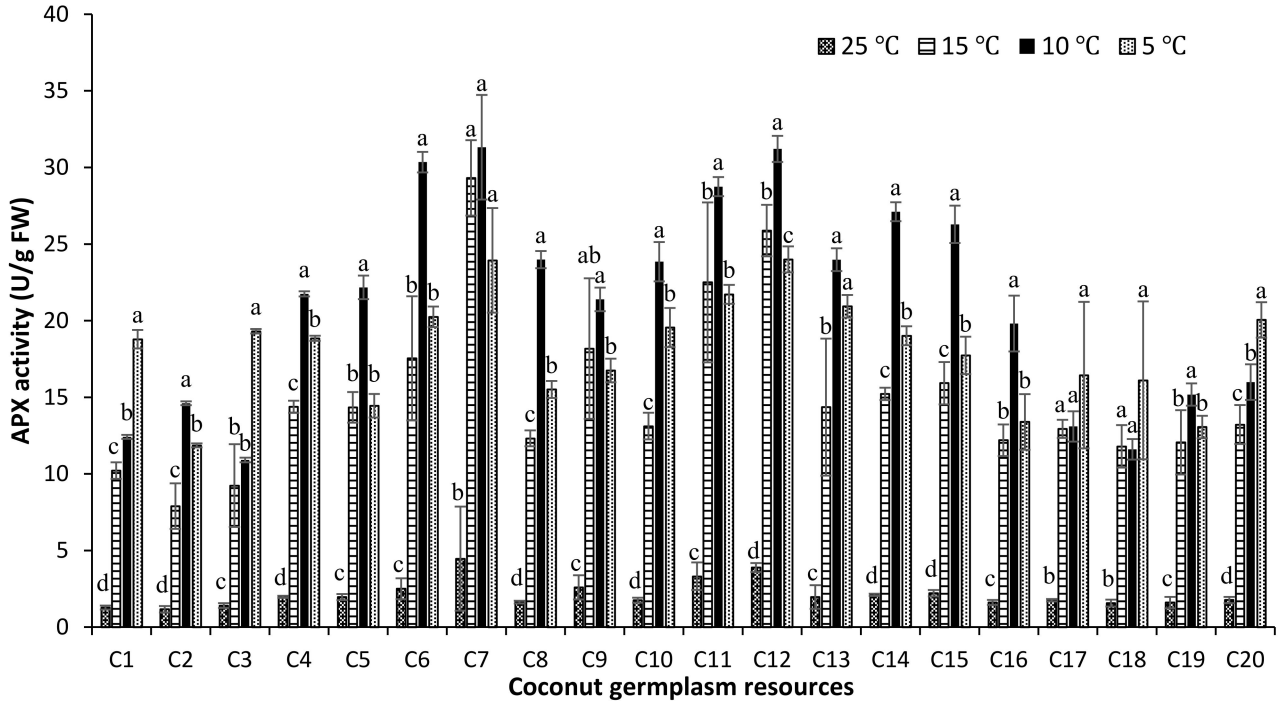

Figure 9. Effects of different low temperature treatments on APX activities of 20 coconut germplasm resources. The data are presented as the mean ± standard deviation (SD). Different lowercase letters indicated significant difference ($p < 0.05$).

The results suggested that the increase of coconut leaf enzyme activities was a defense response to low temperature stress, and the regulation level of APX activity might decrease with the drop of temperature. From a certain point of view, SOD, POD, and CAT activities might also appear similar changes when the temperature is lower than 5 °C.

3.4. Correlation Analysis of Physiological Indexes

Correlation analysis and comparison of seven physiological characteristics (four defense enzyme activities and three osmotic adjustment substance contents) of coconut germplasm resources was shown in Table 2. It is acknowledged that correlation coefficient values near 1 or −1 represent strong relationships between the variables [87]. SOD activity, POD activity, CAT activity, and APX activity manifested significantly ($p < 0.01$) strong positive correlations (Table 2), which might be on account of important components of antioxidant system and defensive enzyme system [88,89], echoing the same change trend of values in the previous analysis. As a reflection of cell membrane lipid peroxidation [90], MDA content showed relatively strong correlation with the four enzyme activities, indicating their functions and effects on ROS [38]. SP content showed a significant positive correlation with SOD and APX activity as well as significant positive relevance with POD activity, verifying the similar change trends of the four. The accumulation of osmolytes is also related to the low temperature tolerance. The SP is among the major osmolytes, and it plays a role in the plant defense enzyme system, including osmotic potential adjustment, carbon frame and energy synthesis, cell metabolism maintaining, and plant cell defense functions under stress [91]. Proline, as a key component of protein in plants, established a significant association to SP content, and both could function in osmotic regulation to prevent cell damage when plants were exposed to cold [29,92]. Additionally, Pro content also performed significantly ($p < 0.01$) moderate correlation with APX activity, which might be related to their ability to scavenge free radicals [93]. The relevance of other indexes was not significant, and the non-significance of some indicators might be due to less contribution to the development or genetic constitution differences [94]. The significant and positive association of the indexes would provide a method to investigate the cold resistance of coconuts and explore physiological mechanism on low temperature.

Table 2. Correlation analysis of physiological indexes of 20 coconut germplasm resources.

Correlation Coefficient	MDA Content	SP Content	Pro Content	SOD Activity	POD Activity	CAT Activity	APX Activity
MDA content	1.00						
SP content	0.18	1.00					
Pro content	0.01	0.76 **	1.00				
SOD activity	0.73 **	0.37 **	0.16	1.00			
POD activity	0.81 **	0.23 *	0.11	0.80 **	1.00		
CAT activity	0.74 **	−0.02	−0.19	0.70 **	0.80 **	1.00	
APX activity	0.41 **	0.57 **	0.57 **	0.59 **	0.59 **	0.29 **	1.00

* Significant correlation at the 0.05 level; ** Significant correlation at the 0.01 level.

3.5. Principal Component Analysis of Physiological Indexes

Principal component analysis is an analysis method for dimensionality reduction evaluation [95], which converts multiple variables into a few representative values to reflect the information comprehensively [96,97]. Principal component analysis helps to understand the whole picture of plant characteristics [98] more simply and conveniently, and contains most of the information to ensure accuracy. It could be seen that the first three principal components contained most of the information, and the cumulative contribution rate reached 88.86% (Table 3), well reflecting the cold resistance of coconut germplasms. POD activity manifested a highest load on the first principal component with 54.52% contribution rate, which accounted for more than half of total contribution value. This indicated that POD activity which played a significant role in performance of coconut

cold resistance could be defined as a representative to evaluate the property of coconuts when exposed to low temperature. Pro content showed the highest value on the second principal component, accounting for 28.91% contribution rate (Table 3). When plants are dehydrated at low temperature, proline plays an important function in osmotic regulation; it may also scavenge free radicals and protect membrane lipids and proteins [99,100]. The multiple function of proline helps to improve the cold resistance of plants and crops [101], which illustrates the representative effect in plant evaluations. Moreover, on the principal component 3, SP content presented the highest value with 5.43% contribution rate. Like proline, SP was also the substance acting as an osmotic adjustment function in plant cells. It could be known that osmotic adjustment substances played an extremely important role in plant response under low temperature. As shown in Table 3, the three physiological indexes of POD activity, Pro content, and SP content could be identified as representatives for markers of coconut cold resistance, providing a reference for further research of coconut resistance cultivation and breeding.

Table 3. Principal component analysis of physiological indexes of coconut germplasm resources.

Physiological Indexes	Principal Component 1	Principal Component 2	Principal Component 3
MDA content	0.43	−0.23	0.31
SP content	0.24	0.54	0.55
Pro content	0.16	0.62	0.05
SOD activity	0.46	−0.07	0.09
POD activity	0.47	−0.16	−0.15
CAT activity	0.39	−0.37	0.10
APX activity	0.38	0.31	−0.75
Contribution rate/%	54.52	28.91	5.43
Cumulative contribution rate/%	54.52	83.43	88.86

3.6. Cluster Analysis of Physiological Indexes

Cluster analysis is an efficient and universal analysis method for germplasm resource evaluation and character appraisal [102,103]. Cluster analysis of seven physiological characters of tested coconut germplasms was shown in Figure 10, and two major clusters were formed based on Euclidean distance. CAT activity formed the first cluster alone, indicating that CAT, to some extent, was not semblable to other indicators. Moreover, the other six indexes constituted the second cluster, explaining that they were analogous in markers of plant resistance. MDA, SP, and Pro content were retrieved, which have been related to the formation and accumulation of ROS and cell osmosis. Moreover, they were clustered into a branch with POD activity and APX activity, both of which functioned to scavenge damaging free radicals. The second cluster comprised this branch as well as the SOD activity branch. From this perspective, the significances of several indicators in coconut cold resistance were presented, and comprehensive analysis was needed to evaluate efficiently.

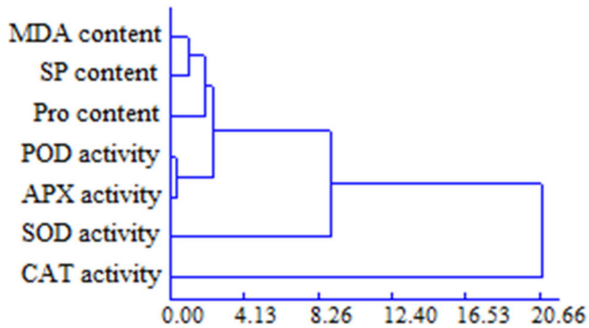

Figure 10. Cluster analysis of physiological indexes of coconut germplasm resources.

3.7. Comprehensive Evaluation

The membership function is a scientific method for comprehensive evaluation of plant cold resistance [104]. Through the comprehensive analysis of physiological indicators related to cold resistance, the values of the membership function were used to express the strength of coconut cold resistance (Table 4), which was more comprehensive and accurate [105]. The osmotic adjustment substances and defense enzyme activities of 20 coconut germplasm resources were determined and compared, and the varieties with stronger cold resistance were explored and investigated. The comprehensive ranking of C10, C2, and C7 was revealed at the forefront (Table 4), indicating that they possessed higher abilities to endure cold environments. Moreover, C12, C16, and C20 were ranked last, suggesting that they were not suitable for cold cultivation and possessed less potential for cold resistance breeding. It was clear that the comprehensive evaluation results were not consistent with the analysis in each indicator, showing that multiple factors should be considered simultaneously in characteristic assessment and evaluation, and there would be limitations in the evaluation of a single index. It was interesting that C10 possessed higher SP and Pro content but not typically higher enzyme activities, indicating that osmotic substances presented active at low temperatures, however, the initiation mechanism of enzyme activities were delayed, which was worth exploring molecular differences in the further study.

Table 4. Membership function values and comprehensive evaluation of physiological indicators of 20 coconut germplasm resources.

Coconut Resources	MDA Content	SP Content	Pro Content	SOD Activity	POD Activity	CAT Activity	APX Activity	Mean Membership Function	Rank
C1	0.14	0.34	0.03	2.45	0.40	0.24	0.19	0.54	13
C2	0.25	1.28	0.82	2.96	1.25	0.58	1.19	1.19	2
C3	0.02	1.19	0.33	1.73	2.56	0.84	0.90	1.08	4
C4	0.09	0.17	0.11	1.04	0.24	0.96	0.38	0.43	17
C5	0.08	0.02	0.08	2.28	0.35	0.24	0.16	0.46	16
C6	0.93	0.50	0.11	3.50	1.11	0.18	0.53	0.98	6
C7	0.45	0.52	0.19	2.46	3.00	0.11	1.27	1.14	3
C8	0.46	0.77	0.28	0.10	0.68	1.61	0.12	0.57	11
C9	0.01	0.19	0.06	2.43	0.45	0.44	0.26	0.55	12
C10	1.13	0.54	1.01	2.90	1.59	0.96	0.25	1.20	1
C11	0.16	0.54	0.39	1.75	2.66	0.43	0.12	0.86	8
C12	0.18	0.05	0.17	1.02	0.10	0.09	0.31	0.27	19
C13	0.02	0.48	0.10	2.23	0.34	0.35	0.07	0.51	15
C14	1.06	0.29	0.09	2.86	1.32	0.53	0.94	1.01	5
C15	0.32	0.37	0.01	1.43	2.84	0.51	0.72	0.89	7
C16	0.33	0.11	0.33	0.47	0.37	0.57	0.13	0.33	18
C17	0.00	0.24	0.06	2.37	0.40	0.46	0.13	0.52	14
C18	0.83	0.28	0.34	1.65	0.72	0.07	0.60	0.64	10
C19	0.30	0.33	0.08	0.89	3.05	0.26	0.49	0.77	9
C20	0.16	0.01	0.21	0.99	0.21	0.15	0.10	0.26	20

In addition, correlation analysis showed that POD activity possessed relatively strong positive association with other three defense enzyme activities and MDA content, ensuring that POD could represent most of the information for resistance assessment. Furthermore, it was consistent with the results that POD activity contributed more than 50% on the first principal component in principal component analysis. Under the comprehensive evaluation of three analytical methods, POD activity, Pro content, and SP content were exhibited as typical physiological indexes to quantify coconut cold resistance. In fact, the findings might provide a conceptual framework for quality assessment and resistance marker of coconut germplasm resources in future research. In this study, the physiological and biochemical measurements in 5 days of treatment of low temperature were implemented, which is the

first stem of genotypes evaluation. Moreover, coconut germplasms in colder environment for longer period should be tested, and the growth parameters, morphology and yield could be studied based on this study. The discovery of coconut cold-resistant germplasms and the simplification of evaluation factors could lay the foundation for coconut introduction, cultivation and variety improvement.

4. Conclusions

As an important economic crop in tropical area, the cultivation, breeding, and processing of coconuts have received extensive attention and with great significance to the development of the coconut industry. The tolerance of coconut to low temperatures plays a decisive role in the introduction and cultivation range, market circulation area, and yield. In this study, the physiological performance and response of 20 coconut germplasm resources to different low temperatures were investigated. The coconut resources C2, C7, and C10 were identified as varieties with strong cold resistance based on a comparison of physiological characteristics such as relative electrical conductivity, osmotic adjustment substance contents, and defense enzyme activities, and could be considered for cold resistant cultivation and breeding. Furthermore, correlation analysis, principal component analysis, and cluster analysis were used for dimensionality reduction and simplification of evaluation factors. The activities of four separate defense enzymes are inducible at low temperatures in different perspectives. POD activity, Pro content, and SP content were determined to be representative indicators in coconut assessment. Their coordination in ROS scavenging deserved further investigation. The results could play a significant role in the research of coconut resistance, which could provide references for the subsequent resistance breeding of coconuts and the resistance research of other tropical palm crops. Meanwhile, this provided a multitude of basic materials for the selection and breeding of cold resistance and had a profound impact on the genetic improvement of freezing tolerance to ensure continuous productivity, so preserving the livelihoods of millions of coconut growers worldwide as well as acclaimed the important source for genetic diversity of coconut in future breeding programs. On the basis of physiological and biochemical changes, coconut performances in colder environment for longer period should be investigated, and the growth as well as yield of coconut germplasms should be explored furthermore.

Author Contributions: C.S. provided experimental ideas and designed experiments. R.Z. conducted the experiment. R.Z. and Z.Y. carried out data processing and analysis and drafted the manuscript. R.Z., H.C. and J.J.J.M. revised the manuscript. H.C. and C.S. supervised and guided the whole experiment. All authors have read and agreed to the published version of the manuscript.

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