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Wood Vinegar Impact on the Growth and Low-Temperature Tolerance of Rapeseed Seedlings

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Abstract: Low temperature seriously affects the growth of crops and poses a huge threat to food production. The application of wood vinegar can be used as a cost-effective and environmentally friendly strategy to promote crop growth and enhance stress resistance, and the physiological resistance to low-temperature stress of rapeseed still needs further research. The present study investigated the effects of spraying wood vinegar on the growth, photosynthesis, osmotic adjustment, and antioxidant enzymes of rapeseed seedlings under low-temperature stress. The results showed that spraying wood vinegar at normal temperature reduced the stomatal conductance but increased the leaf area and total biomass of rapeseed and enhanced stomatal density and water use efficiency. The leaf area and total biomass of rapeseed sprayed with wood vinegar at low temperature improved by 22% and 31%, respectively, and stomatal density and water use efficiency increased by 14% and 83%, respectively, and intercellular CO_2 concentration and stomatal conductance were reduced by 9% and 41%, compared to the low-temperature, respectively. Besides, the application of wood vinegar liquid improved the proline, soluble protein, and soluble sugar content of leaves by 208%, 38%, and 115%, respectively, and the activity of superoxide dismutase increased by 27%, the content of malondialdehyde decreased by 46%, compared to the low-temperature. Spraying wood vinegar could alleviate low-temperature stress by improving the anti-oxidant enzyme content and osmoprotectants, reducing the stomatal conductance, and enhancing water use efficiency. These results provide new insights for wood vinegar to relieve the low-temperature stress of rapeseed, and this strategy can be used for low-temperature rapeseed cultivation and management, and benefit farmers' plant profit.

Keywords: wood vinegar; *Brassica napus*; low temperature; stomatal density; anti-oxidant enzymes; osmoprotectants

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1. Introduction

Wood vinegar is an organic liquid obtained by condensation and separation of the gas produced during the pyrolysis and carbonization of biomass. It is a mixture of various organic substances [1], mainly acids and phenols, as well as aldehydes, ketones, alcohols, esters, and trace elements such as potassium, calcium, magnesium, iron [2]. Recently, wood vinegar has been used as a foliar fertilizer and plant growth regulator in agricultural production [3]. Previous research studies on rapeseed have shown that wood vinegar has the most obvious effect on root development, leaf area expansion, and biological yield [4]. As natural antioxidants, organic acids and phenols in wood vinegar also affect the physiological activities of plants, can promote plant growth and development under adversity, and effectively enhance plant resistance [5,6]. Under normal conditions, wood vinegar can significantly improve the protective enzyme activities of rice, tobacco, and rapeseed seedlings [6,7]. Under low-temperature stress, wood vinegar can improve the growth of various indicators of rice seedlings, promote the increase of osmotic adjustment substances, enhance the antioxidant activity and root vitality of rice seedlings, and raise the cold resistance of rice seedlings [8].

Low temperature is one of the main abiotic stresses that restrict crop growth. It not only affects the geographic distribution of crops but also directly affects crop growth and development and final yield [9]. Plants subjected to low-temperature stress can reduce the plant growth rate, leaf wilting, and tissue death [10]. The cell membrane is the primary site to observe the response to low-temperature stress [11]. Low-temperature stress reduces the ability of plants to use oxygen, and excess oxygen during metabolism is transferred through electron transfer to form reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide, hydroxyl radicals, and singlet oxygen [12]. When the large amount of active oxygen produced in the plant exceeds the plant's scavenging ability, ROS begins to attack biological macromolecules, such as membrane lipids, nucleic acids, and proteins. Lipid membrane peroxidation will produce malondialdehyde, causing damage to the biological membrane system. In turn, the membrane structure and function will destroy, resulting in a decrease in photosynthetic products and abnormal metabolism [13].

To enhance stress resistance, plants have evolved a complex antioxidant system to resist external environmental stress, including superoxide dismutase (SOD), catalase (POD), peroxidase (CAT), and other free radical scavengers [14]. As the first line of defense for membrane protection, SOD's main function is to convert the generated ROS into H_2O_2 and then decompose H_2O_2 into H_2O and O_2 through POD and CAT [15]. In addition, various organic and inorganic substances (such as proline, soluble sugar, and soluble protein) accumulate in plant cells for osmotic adjustment in response to various abiotic stresses, reducing cell osmotic potential and alleviating the effects of adversity stress [16].

Rapeseed (*Brassica napus* L.) is one of the four major oil crops globally, and it is also a widely planted overwintering oil crop in China. It occupies an important position in China's agricultural production [17]. The largest rapeseed production area in China is mainly located in the Yangtze River Basin, and low temperature and chilling damage are common winter weather disasters in the Yangtze River Basin, which has a greater risk of reducing rapeseed production [18]. In addition, the low temperature and snow in extreme weather years have caused different degrees of frost damage to rapeseed at the seedling stage in many provinces in China, and even no harvest [19]. Therefore, research and prevention of low temperature and freezing damage are of great significance to the development of rapeseed production. Plant growth regulators can effectively regulate plant growth, development, and adaptability to the environment. Previously, various plant growth regulators were used in agricultural production to effectively regulate and control the growth and development of crops, enhance stress resistance, increase yield and improve quality [20]. In recent years, some researchers have found that wood vinegar has a good effect as a plant growth regulator [2]. Due to its environment-friendly, low cost, and positive effect on plant growth, it has gradually attracted the attention of agricultural researchers in different fields.

Previously, many studies have been conducted on the low temperature freezing damage and resistance of rapeseed, however, the research on the effect of wood vinegar on improving the ability of rapeseed to resist low temperature has not been systematically investigated. We hypothesize that wood vinegar could enhance low-temperature stress resistance at the seeding stage of rapeseed. This study aimed to elaborate on the effects of foliar spraying of wood vinegar on the growth of rapeseed plants, photosynthesis characteristics, and osmotic adjustment under low-temperature stress, and provide new information on rapeseed resistance to low temperature under the application of wood vinegar.

2. Materials and Methods

2.1. Test Site, Materials, and Soil Characteristics

The experiment was carried out in the laboratory of Huazhong Agricultural University from October to November 2020. The silty loam soil was used, and the weight of each pot was 0.9 kg. The basic chemical properties of soil prior to the experiment had a soil pH of 6.0, organic matter 7.5 g kg^{-1}, ammonium nitrogen 1.5 mg kg^{-1}, nitrate-nitrogen 7.9 mg kg^{-1}, available phosphorus 8.4 mg kg^{-1}, available potassium 113 mg kg^{-1}, sulfur 170 mg kg^{-1}. The test material was the hybrid rapeseed Huayouza 9, which is widely grown in the Yangtze River Basin, and was provided by Shengguang Seed Industry Co., Ltd. (Wuhan, China). Wood vinegar came from the smoke of poplar wood charcoal and was provided by Hubei Chutian Biomass Energy Development Co., Ltd. (Wuhan, China). The laboratory used a gas chromatography-mass spectrometer to determine the effective ingredients of wood vinegar up to 159 kinds [21].

2.2. Experimental Design

The seedlings were grown in pots, and 0.6 g compound fertilizer (N15-P15-K15) was applied to each pot containing nitrogen: 0.1 g kg^{-1}, phosphorus: 0.1 g kg^{-1}, and potassium: 0.1 g kg^{-1} soil. Five seeds per pot were sown on 19 October 2020, set seedlings at the two-leaf stage, and retained one robust seedling with the same growth in each pot. After the three-leaf stage, plants were transferred to a smart temperature-light incubator (GDN-400, Ledian, Ningbo, China). The incubator conditions were set to normal temperature (25 °C during the day, 20 °C at night) and low temperature (10 °C during the day, 5 °C at night), the illumination was 150 μmol m^{-2} s^{-1}, and the ratio of light to dark was L:D = 14:10 h.

A total of four treatments were set up in the experiment, namely, normal temperature (CK), normal temperature + wood vinegar (CK + M), low temperature (LT), and low temperature + wood vinegar (LT + M). According to the preliminary test results of this laboratory, a 400-fold diluted wood vinegar was used to spray the rapeseed leaf surface, tween 80 at 0.1% was used as a surfactant, and sprayed twice during the three-leaf period (11 November, 16 November) of the rapeseed, with an amount of 5 mL per plant, the control group was sprayed with the same amount of distilled water. Each treatment was repeated 20 times and arranged randomly.

On 30 November, forty-two days after sowing, rapeseed seedlings were samples to investigate the morphological, physiological, and photosynthetic indicators.

2.3. Morphological Indicators

To investigate the morphological indicators, five plants for each treatment were selected. The leaf area was scanned and imaged with an Epson V800 scanner at a resolution of 300 dpi, and the image was analyzed with Image J to calculate the leaf area. The leaf thickness was determined using a thickness meter (SYS-YHD-2, Saiyasi, China). The dry matter weight was determined by selecting 5 rapeseeds and roasting them to a constant weight.

Stomatal density and number measurement samples were collected at 10:00 in the morning, and a layer of transparent nail polish was evenly coated on the leaf epidermis of the fully unfolded leaf, dried, and then peeled off with scotch tape and placed on a glass slide. Observe the stomatal density under the 20-fold objective lens of an inverted fluorescence microscope (Olympus IX71, Corporation, Tokyo, Japan), and measure the length (L) and width (W) of the stomata under a 60-fold objective lens. Apertures were measured randomly from six stomata on the same specimens using ImageJ software [22], and each leaf was randomly observed six times.

2.4. Physiological Indicators

Used Li-Cor 6800 (Li-COR Inc., Lincoln, NE, USA) portable photosynthesis instrument to measure the gas exchange rate of rapeseed leaves, control the leaf chamber light intensity to 1000 μmol m^{-2} s^{-1}, and process the leaf chamber temperature at the normal temperature set to 25 °C, set low-temperature treatment to 10 °C, the air vapor pressure deficit (VPD) was 1.2–1.8 kPa and repeated six times [23]. The measurement indicators include net photosynthetic rate (*A*), intercellular CO_2 concentration, stomatal conductance (*gs*), and transpiration rate, and then calculate the water use efficiency (WUE), the calculation formula is WUE = A/gs.

Soluble protein was determined using Coomassie brilliant blue with bovine serum albumin as the standard [24]. The chlorophyll content was measured by the 95% ethanol extraction method [25]. Malondialdehyde was determined by the thiobarbituric acid method [26]. Proline was measured by the ninhydrin method [27]. Soluble sugars were determined by the anthrone colorimetric method [28]. SOD, POD, CAT, H_2O_2, and O_2^- were measured by WST-1 method, colorimetric, UV-visible spectrophotometry, Spectrophotometry, and Hydroxylamine oxidation, respectively [29], and reagents were provided by the Nanjing Jiancheng Biotechnology Co., Ltd. (Nanjing, China).

2.5. Data Processing and Analysis

SPSS 25.0 (SPSS Inc., Chicago, IL, USA) software was used for the one-way analysis of variance, and a two-way analysis of variance was performed for the interaction between low temperature and wood vinegar. The difference analysis between each treatment was based on the 0.05 level Least significant difference method (LSD). Microsoft Excel 2019 software was used for mapping.

3. Results

3.1. The Effect of Wood Vinegar on the Growth of Rapeseed Seedlings under Low-Temperature Stress

3.1.1. Biomass

The results showed that the fresh weight, root dry weight, above-ground biomass, and total biomass of rapeseed treated with CK + M increased by 10%, 6%, 14%, and 12%, respectively, compared to CK. Low-temperature stress significantly decreased fresh weight, root dry weight, above-ground biomass, and total biomass of rapeseed treated with LT were reduced by 55%, 44%, 47%, and 46%, respectively, compared to CK (Figure 1). The application of wood vinegar significantly increased the fresh weight, root dry weight, above-ground biomass, and total biomass of rapeseed treated with LT + M increased by 22%, 33%, 30%, and 31%, respectively, compared to LT. These results suggest that the rapeseed sprayed with wood vinegar could promote the dry matter accumulation of seedlings at normal temperature and low temperature, and the increase was greater under low-temperature stress, especially the enhancement of root dry weight at low temperature.

3.1.2. Agronomic Traits

The finding of the current study showed that under normal temperature conditions, the leaf area of rapeseed treated with CK + M increased by 14% compared to CK. Low-temperature stress significantly inhibited the growth of rapeseed with a reduction in the total number of leaves, the number of green leaves, and leaf area of rapeseed under LT by 47%, 47%, and 76%, respectively, while the leaf thickness increased by 88% compared to CK (Table 1). The leaf area of rapeseed treated with LT + M increased by 22% compared to LT, but it has little effect on the number of total leaves, the number of green leaves, and leaf thickness of rapeseed. The current findings indicate that spraying wood vinegar at normal temperature and low temperature can promote the growth and development of seedlings by improving leaf area, and the effect of promoting leaf area growth under low-temperature stress was better.

Table 1. Effects of wood vinegar on the growth of rapeseed seedlings under low-temperature stress.

Treatment	Number of Total Leaves	Number of Green Leaves	Leaf Area (cm^2)	Leaf Thickness (mm)
CK	7.5 ± 0.2 a	5.7 ± 0.3 a	2833 ± 78 b	0.31 ± 0.01 b
CK + M	8.0 ± 0.3 a	6.0 ± 0.2 a	3221 ± 110 a	0.32 ± 0.01 b

Table 1. *Cont.*

Treatment	Number of Total Leaves	Number of Green Leaves	Leaf Area (cm^2)	Leaf Thickness (mm)
LT	4.0 ± 0.0 b	3.0 ± 0.0 b	687 ± 56 d	0.62 ± 0.03 a
LT + M	4.2 ± 0.2 b	3.2 ± 0.2 b	836 ± 19 c	0.63 ± 0.02 a

Note: According to the LSD test, the lowercase letters after the numbers in each column of the table indicate significant differences ($p < 0.05$), Error bars are ± SE (n = 5). CK, normal temperature; CK + M, normal temperature + wood vinegar; LT, low temperature; LT + M, low temperature + wood vinegar.

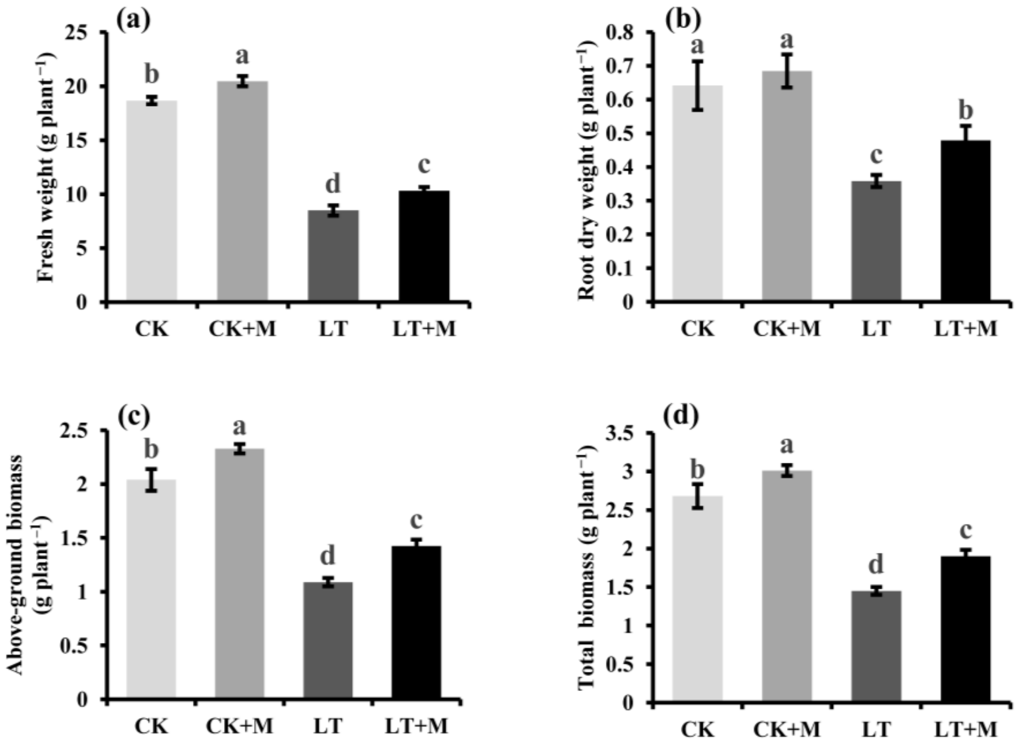

Figure 1. Effects of wood vinegar on the biomass of rapeseed seedlings fresh weight (**a**), root dry weight (**b**), above-ground biomass (**c**), and total biomass (**d**) under low-temperature stress. According to the LSD test, the lowercase letters in the figure indicate significant differences ($p < 0.05$). Error bars are ± SE (n = 5). CK, normal temperature; CK + M, normal temperature + wood vinegar; LT, low temperature; LT + M, low temperature + wood vinegar.

3.2. Effects of Wood Vinegar on Photosynthetic Characteristics and Stomatal Density of Rapeseed Seedlings under Low-Temperature Stress

3.2.1. Stomatal Density and Aperture

The density and aperture of stomata were measured to evaluate the role of wood vinegar on stomata traits under low-temperature stress. The results showed that the stomatal density of rapeseed treated with CK + M increased by 6% under normal temperature conditions compared to CK, which had little effect on stomatal aperture. Low-temperature stress significantly reduced the stomatal density and stomatal aperture of rapeseed seedlings. Figure 2 shows that the two indexes of rapeseed under LT decreased by 20% and 13% compared to CK, respectively. The wood vinegar spray treatment mainly relieved the inhibitory effect of low temperature on the growth of seedlings by enhancing the stomatal density. The stomatal density of the LT + M treatment increased by 14% compared to LT, and the effect on the stomatal aperture was still not significant. It shows that spraying wood vinegar at normal temperature and low temperature can enhance the stomatal density on the leaf surface, combined with the increase of the unit leaf area, we speculated that it might improve the overall carbon assimilation ability of the plant, thereby promoting the accumulation of dry matter in the seedlings.

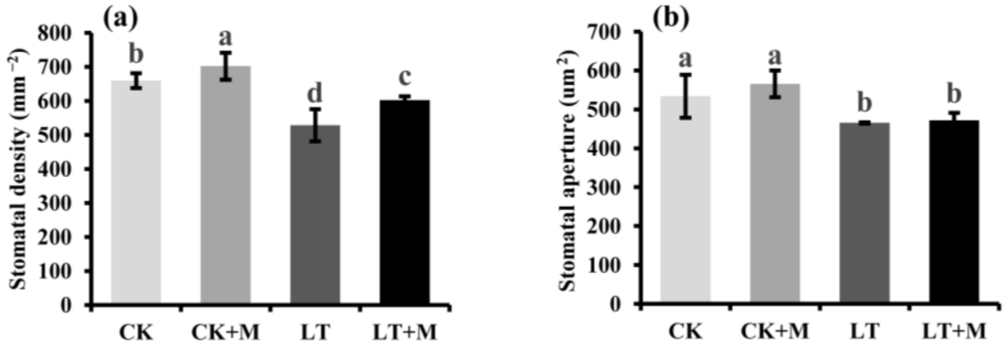

Figure 2. Effects of wood vinegar on the stomatal density and aperture of rapeseed seedlings stomatal density (**a**) and stomatal aperture (**b**) under low-temperature stress. Error bars are ± SE (n = 6). According to the LSD test, the lowercase letters in the figure indicate significant differences ($p < 0.05$). CK, normal temperature; CK + M, normal temperature + wood vinegar; LT, low temperature; LT + M, low temperature + wood vinegar.

3.2.2. Photosynthetic Capacity and Water Use Efficiency

To further explore the effect of wood vinegar on photosynthesis, we use a photosynthetic instrument to determine the index of photosynthesis. The results showed that under normal temperature conditions, compared to CK, the application of wood vinegar treatment has no effect on leaf chlorophyll content and net photosynthetic rate but reduces stomatal conductance, decreasing transpiration rate, and finally increasing water use efficiency by 16%. Low-temperature stress significantly reduced the photosynthetic capacity of rapeseed seedlings (Table 2). The net photosynthetic rate, transpiration, stomatal conductance, and chlorophyll content were decreased by 31%, 62%, 37%, and 31%, respectively, while the intercellular CO_2 concentration increased significantly, which raised the water use efficiency by 14% compared to CK. Compared to LT, the net photosynthetic rate of LT + M tends to increase, and the intercellular CO_2 concentration was 9% lower than that of LT. Besides, based on significantly improving the stomatal density, the stomatal conductance was reduced by 41%, and the water use efficiency was significantly increased by 55%. Therefore, spraying wood vinegar under low-temperature stress can further decrease the stomatal opening and reduce the transpiration rate to deal with the adverse effects of adversity while maintaining the net photosynthetic rate.

Table 2. Effects of wood vinegar on photosynthetic characteristics of rapeseed seedlings under low-temperature stress.

Treatment	Chlorophyll Content (mg/g)	Transpiration Rate (mmol m^{-2} s^{-1})	Net Photosynthetic Rate (μmol m^{-2} s^{-1})	Intercellular CO_2 Concentratio (μmol mol^{-1})	Stomatal Conductance (mol m^{-2} s^{-1})	Water Use Efficiency (μmol mol^{-1})
CK	1.5 ± 0.1 a	13.0 ± 0.6 a	25.8 ± 1.0 a	326 ± 8 ab	1.5 ± 0.2 a	1.72 ± 0.09 c
CK + M	1.5 ± 0.1 a	11.6 ± 0.5 b	25.9 ± 0.9 a	322 ± 7 bc	1.3 ± 0.2 a	1.99 ± 0.07 b
LT	1.0 ± 0.1 b	4.9 ± 0.6 c	17.7 ± 0.8 b	339 ± 9 a	0.9 ± 0.1 b	1.96 ± 0.06 b
LT + M	1.2 ± 0.1 b	3.8 ± 0.5 c	18.2 ± 0.5 b	312 ± 4 c	0.6 ± 0.0 c	3.03 ± 0.12 a

Note: According to the LSD test, the lowercase letters after the numbers in each column of the table indicate significant differences ($p < 0.05$), (n = 4). CK, normal temperature; CK + M, normal temperature + wood vinegar; LT, low temperature; LT + M, low temperature + wood vinegar.

3.3. The Effect of Wood Vinegar on Osmotic Adjustment of Rapeseed Seedlings under Low-Temperature Stress

The results showed that under normal temperature conditions, the proline, soluble protein, and soluble sugar content of rapeseed seedlings treated with CK + M increased by 62%, 41%, and 89%, respectively, compared to CK. Low-temperature stress cause a significant increase in the proline and soluble protein contents of rapeseed seedlings but had no significant effect on the soluble sugar content. Figure 3 shows that the proline, soluble protein, and soluble sugar content of rapeseed seedlings treated with LT increased by 237%, 21%, and 14%, respectively, compared to CK. After spraying wood vinegar, the content of osmotic adjustment substances further improved. The proline, soluble protein, and soluble sugar content of seedlings treated with LT + M increased by 208%, 38%, and 115%, respectively, compared to LT. It shows that spraying wood vinegar can significantly enhance the content of osmotic adjustment substances and improve the resistance of rapeseed to low-temperature stress.

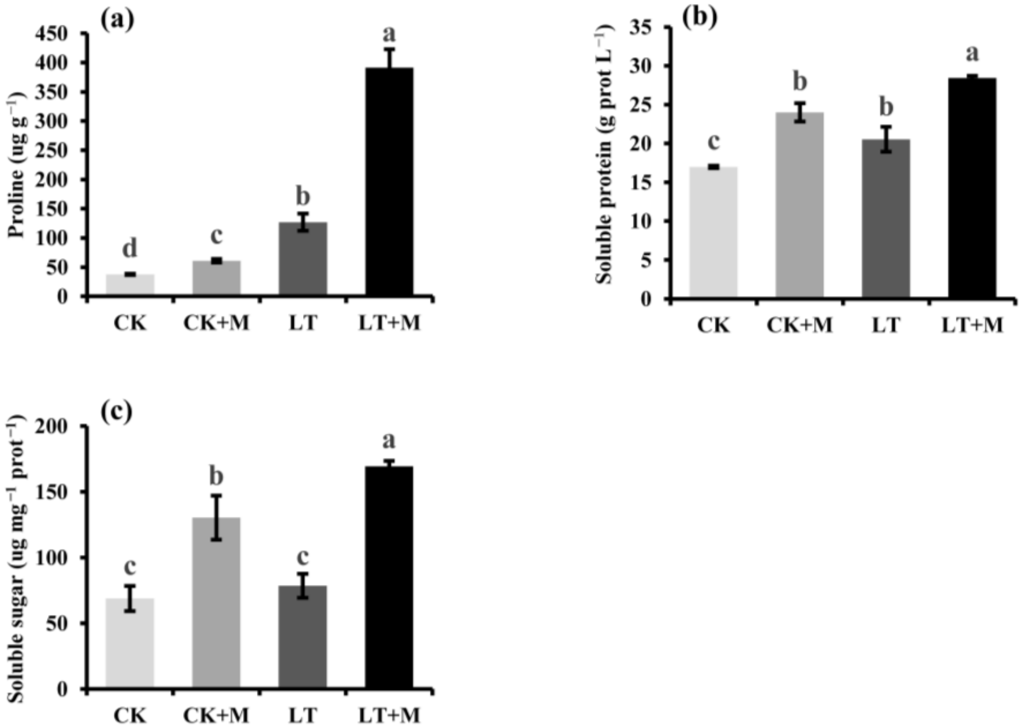

Figure 3. Effects of wood vinegar on osmotic adjustment substances in rapeseed seedlings proline (**a**), soluble protein (**b**), and soluble sugar (**c**) under low-temperature stress. Error bars are ± SE (n = 3). According to the LSD test, the lowercase letters in the figure indicate significant differences ($p < 0.05$). CK, normal temperature; CK + M, normal temperature + wood vinegar; LT, low temperature; LT + M, low temperature + wood vinegar.

3.4. Effect of Wood Vinegar on Malondialdehyde and Antioxidant Enzyme Activities of Rapeseed Seedlings under Low-Temperature Stress

3.4.1. Malondialdehyde

The findings of the study revealed that under normal temperature conditions, the malondialdehyde content of rapeseed treated with CK + M was 40% lower than that of CK (Figure 4). Low-temperature stress caused a significant increase in the malondialdehyde content of rapeseed, indicating that the low-temperature environment increased the lipid peroxidation of rapeseed seedlings and exerted certain damage. Compared to LT, LT + M reduced malondialdehyde by 46%, and compared to CK, it decreased by 32%. These results indicate that spraying wood vinegar's effect on alleviating the damage of low-temperature stress on the cell membrane was very strong, and it can effectively inhibit the membrane peroxidation of rapeseed leaves.

3.4.2. Reactive Oxygen Species

The results showed that the content of H_2O_2 and O_2^- in rapeseed treated with CK + M was 28% and 34% lower than CK under normal temperature. After spraying wood vinegar, the content of H_2O_2 and O_2^- in LT + M was 25% and 17% lower than that in LT respectively. It showed that spraying wood vinegar could reduce the content of H_2O_2 and O_2^-, alleviate the damage of reactive oxygen species to rapeseed cells, and improve the resistance of rapeseed to low-temperature stress.

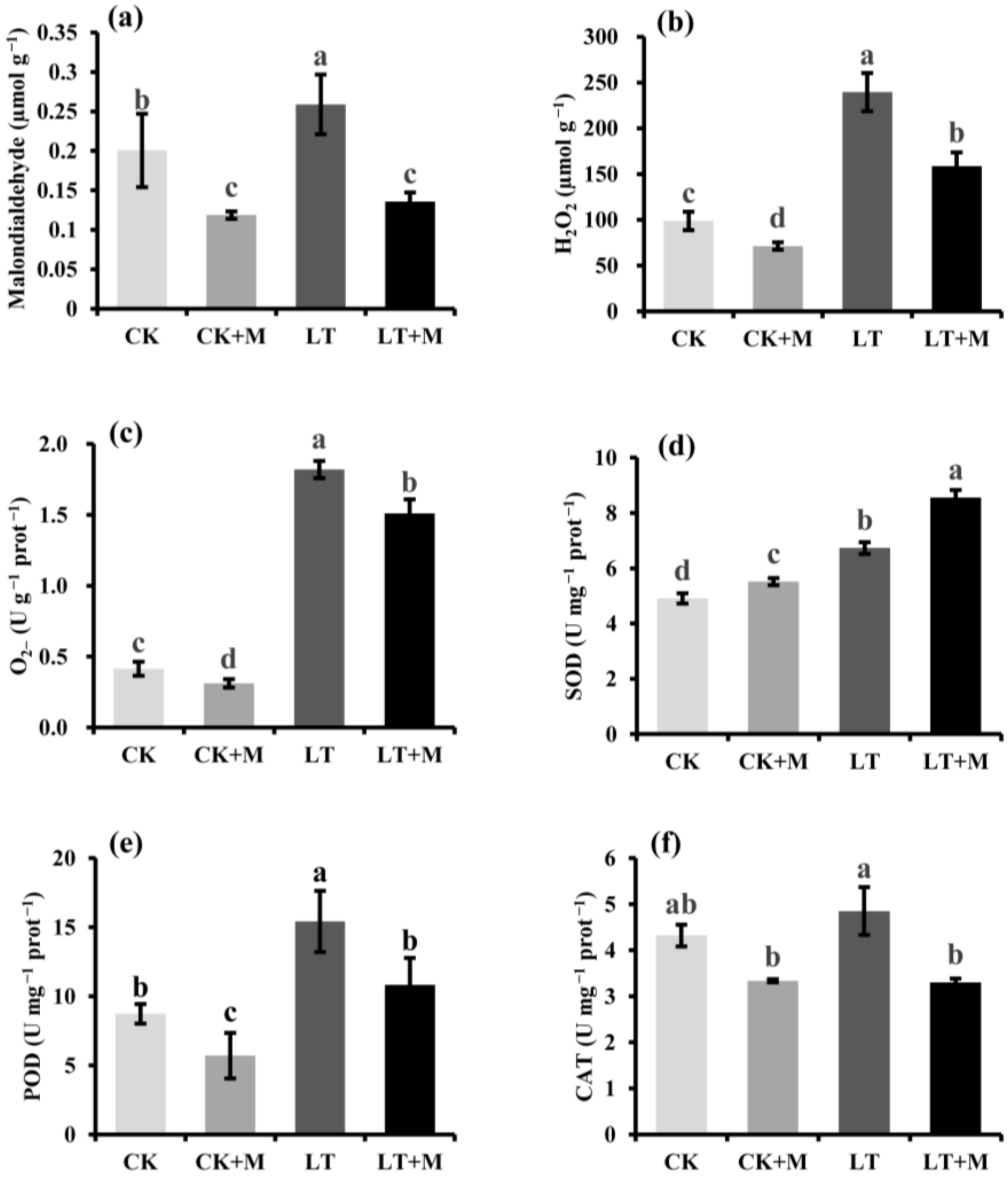

Figure 4. Effects of wood vinegar on malondialdehyde and antioxidant enzyme activities of rapeseed seedlings malondialdehyde (**a**), H_2O_2 (**b**), O_2^- (**c**), SOD (**d**), POD (**e**), and CAT (**f**) under low-temperature stress. Error bars are ± SE (n = 3). According to the LSD test, the lowercase letters in the figure indicate significant differences ($p < 0.05$). CK, normal temperature; CK + M, normal temperature + wood vinegar; LT, low temperature; LT + M, low temperature + wood vinegar. SOD, superoxide dismutase. POD, catalase. CAT, peroxidase.

3.4.3. Antioxidant Enzymes Activities

The results showed that under normal temperature conditions, the activity of SOD in rapeseed treated with CK + M increased by 12% compared to CK, but the activities of POD and CAT decreased by 35% and 30%, respectively. After spraying wood vinegar, the SOD activity of LT + M increased by 27% compared to LT, but the activities of POD and CAT decreased by 23% and 30%. It showed that the spraying of wood vinegar solution further enhanced the ability of superoxide disproportionation to H_2O_2 produced under low-temperature stress by raising the SOD activity of rapeseed, thereby effectively improving the resistance of rapeseed to low-temperature stress.

3.5. Effects of the Interaction of Low Temperature and Wood Vinegar on the Physiological Indexes of Rapeseed

Two-factor analysis of variance showed that low temperature and wood vinegar treatment has significant effects on proline, soluble sugar, and SOD, which induced plants to increase stress resistance, and thus have significant interactions with proline, soluble sugar, and SOD (Table 3; Figure 5). It shows that these three indicators can be used as the characteristic physiological parameters to measure the wood vinegar response to relieve the low-temperature stress of rapeseed.

Table 3. Variance analysis of the influence of low temperature and wood vinegar treatment and their interaction on the physiological indexes of rapeseed.

Physiological Indexes	Low Temperature	Wood Vinegar	Low-Temperature × Wood Vinegar
Proline	**	**	**
Soluble sugar	*	**	**
SOD	**	**	*

Note: * $p < 0.05$. ** $p < 0.01$.

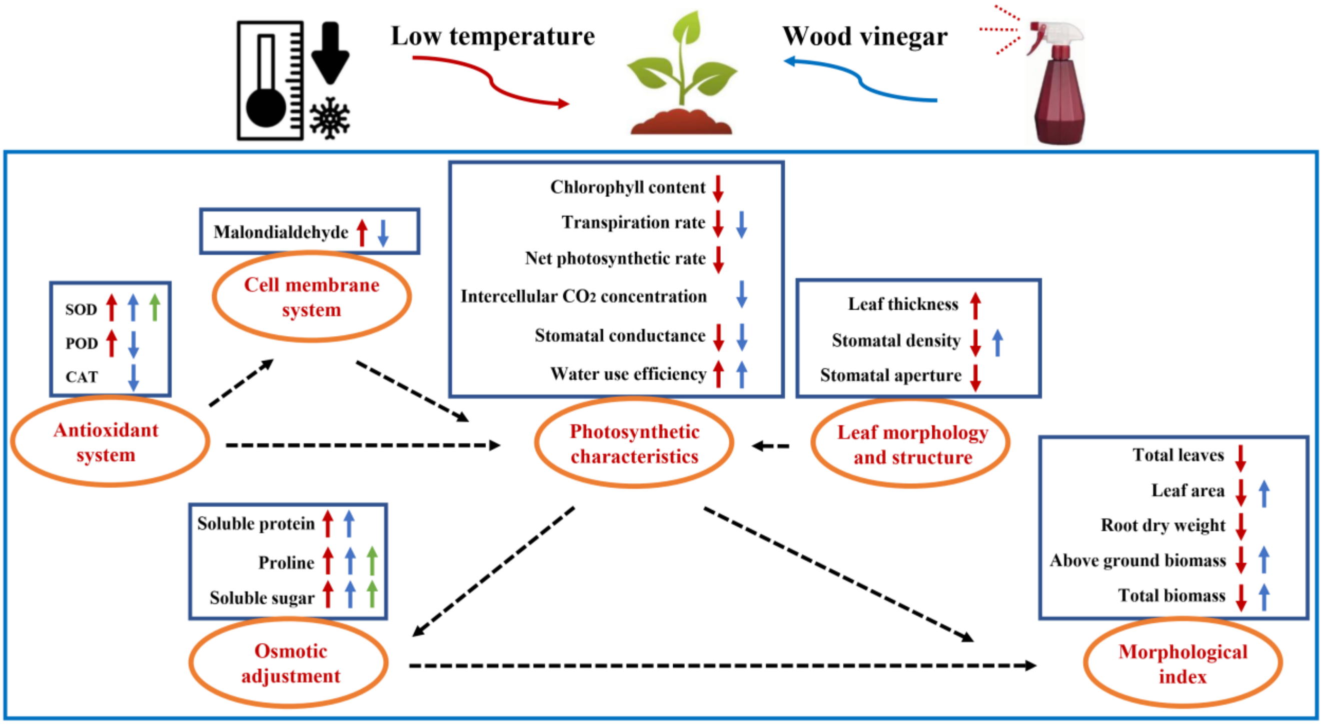

Figure 5. Comprehensive analysis of differences between low temperature and wood vinegar on various indicators of rapeseed. The red, blue, and green arrows represent significant differences in low-temperature treatment, wood vinegar treatment, and low temperature + wood vinegar treatment ($p < 0.05$). Upward means increase, and downward means decrease. SOD, superoxide dismutase. POD, catalase. CAT, peroxidase.

4. Discussion

4.1. Wood Vinegar Can Promote the Growth of Rapeseed Seedlings under Low-Temperature Stress

The effects of low-temperature stress on plants were mainly reflected in enzyme activity, membrane system, and cell dehydration, etc. As the activity of photosynthetic-related enzymes was reduced, the photosynthetic rate of plants was also decreased [30], thereby slowing down plant growth rate leading to insufficient organ development and a significant decrease in biomass [31]. In this experiment, low-temperature stress significantly reduced the photosynthetic rate of rapeseed and increased intercellular carbon dioxide concentration. Spraying wood vinegar can effectively alleviate the hindrance of low temperature and significantly promote the expansion of rapeseed leaf area and dry matter accumulation (Table 1). The experimental study [21] showed that wood vinegar contains at least 159 organic substances, including eight categories (phenols 43%, organic acids 27%, ketones 11%, esters 4%, alkane compounds 3%, furan derivatives 1%, aldehydes 2% and nitrogen compounds 1%), of which acids, phenols, crotonolactone, furan, and its derivatives and other substances have the effect of promoting plant growth. Related studies have shown that the acids and phenols in wood vinegar are substances with high biological activity [1]. The research findings on peppers show that phenolic substances, especially dihydric phenols and polyphenols, can significantly promote the growth of peppers at low concentrations [32]. Research on rapeseed has shown that furan and its derivatives may stimulate plant growth [33]. In summary, wood vinegar contains a variety of substances that are beneficial to crop growth. Under appropriate concentrations, these substances can synergistically produce a good comprehensive effect [7], effectively promoting plant growth and dry matter accumulation.

4.2. Wood Vinegar Can Coordinate Low-Temperature Resistance and Photosynthesis by Significantly Increasing Stomatal Density and Reducing Stomatal Conductance

Leaves are the main place for the photosynthesis of plants, and the density and aperture of stomata on the leaf epidermis directly affect photosynthesis [34]. Previous studies have shown that the factors affecting plant photosynthesis can be divided into stomatal factors and non-stomatal factors [35], among which stomatal factors mainly include stomatal density and stomatal aperture, and non-stomatal factors mainly include temperature, carbon dioxide, and light intensity. When the intercellular CO_2 concentration and stomatal conductance decreased simultaneously, the reduction in the net photosynthetic rate was mainly caused by stomatal factors. When the net photosynthetic rate decreases with the increase of intercellular CO_2 concentration, the main limiting factor was non-stomatal factors [36]. Our study results showed that the net photosynthetic rate and stomatal conductance of rapeseed leaves were significantly reduced under low-temperature stress, and the intercellular CO_2 concentration increased (Table 2; Figure 2). Besides, the stomatal density and aperture were also significantly decreased, indicating that under low-temperature stress, the reason for the reduced net photosynthetic rate of rapeseed was the combined effect of non-stomatal factors and stomatal factors. However, after spraying wood vinegar under low-temperature conditions, the leaf stomatal density improved significantly, not only the stomatal conductance was further decreased, but the intercellular CO_2 concentration was also reduced, and the net photosynthetic rate showed an increasing trend. It shows that wood vinegar can significantly decrease leaf transpiration by further reducing stomatal conductance and improving low-temperature resistance. Based on the results of these studies, we believe that the application of wood vinegar under low-temperature stress decreases the stomatal conductance, and transpiration rate, and at the same time increases the stomatal density and expands the leaf area, to a certain extent, the photosynthesis and dry matter accumulation capacity of plants were enhanced.

4.3. Wood Vinegar Mainly Enhances the Low-Temperature Resistance of Rapeseed by Greatly Increasing the Content of Proline

Osmotic adjustment substances such as proline, soluble sugar, and soluble protein in plants were closely related to cold resistance [37,38], and they mainly maintain intracellular turgor pressure by reducing the osmotic potential to maintain moisture and the normal progress of various physiological processes of cells. Proline is one of the components of plant protein, which is widely present in plants in a free state. Previous studies have shown that, as an osmotic regulator in plant cytoplasm, proline is first due to its extremely strong hydrophilicity, which can stabilize the metabolic processes in protoplast colloids and tissues, decrease the freezing point, and play an important role in preventing cell dehydration [39]. On the other hand, proline also has the effect of promoting plant growth, maintaining the conformation of various enzymes in plants, improving the solubility of proteins in water, reducing cell acidity, detoxifying ammonia, and regulating cell redox potential as an energy reservoir [40]. Under normal conditions, the level of soluble sugar content was closely related to the carbon and nitrogen level of plants and healthy growth [41]. The possible reasons for improving the cold resistance of tissues are: One is to reduce the freezing point through the accumulation of sugar and enhance the water retention capacity of cells. The second is through sugar metabolism, to produce other protective substances and energy. The third is to protect the cell's vital substances and biofilm [41]. Soluble protein can increase the amount of bound water in the cell and improve the water retention capacity of the cell. Besides, it can enhance the concentration in the cytoplasm to reduce the damage of low temperature [14].

The results of this experiment showed that spraying wood vinegar can increase the proline, soluble protein, and soluble sugar content of rapeseed seedlings under normal temperature and low-temperature stress. But after using wood vinegar under low-temperature stress, the increase of proline was as high as 208%, which was significantly higher than 62% under normal temperature conditions and much higher than the improvement of soluble protein and soluble sugar content (Figure 3). Studies on blueberries have also found that wood vinegar can enhance the proline and soluble sugar content of plants under normal conditions [42]. These results indicate that the application of wood vinegar also has an osmotic adjustment effect under normal conditions, and through the maintenance of enzyme activity, the realization of carbon and nitrogen balance, and the above-mentioned comprehensive regulation of stomata, it coordinates various physiological and biochemical processes of plants and comprehensively promotes the healthy growth and dry matter accumulation of plants. Under low-temperature adversity conditions, the energy pool can be regulated by greatly increasing the proline content, and the plant's stress-resistant growth can be promoted. Besides, together with the high soluble sugars and soluble proteins, the plant's low-temperature resistance can be improved synergistically.

4.4. Wood Vinegar Can Greatly Increase the Activity of the SOD Enzyme to Enhance the Ability of Rapeseed to Resist Low Temperature

Malondialdehyde is the end product of membrane lipid peroxidation and can be used as an important indicator for studying the degree of membrane lipid loss and the strength of plant resistance [43,44]. Under low-temperature conditions, plants will generate a large amount of toxic hydrogen peroxide, hydroxyl free radicals, singlet oxygen, and other active oxygen free radicals [13]. Free radicals can cause the peroxidation of plant membrane lipids and damage the integrity of the membrane structure [12]. Studies have shown that the malondialdehyde content of plants rises sharply under low-temperature stress, and their osmotic adjustment ability is reduced, which seriously affects the physiological metabolism of plants [45]. This study shows that wood vinegar treatment can effectively decrease the malondialdehyde content of rapeseed leaves at normal temperature and low temperature, and the content of malondialdehyde after wood vinegar treatment at low temperature is equivalent to the level under normal temperature control (Figure 4), indicating wood vinegar treatment under stress can better alleviate the cell membrane damage of rapeseed seedlings, reduce the accumulation of active oxygen and chlorophyll decomposition, and enhance the cold resistance of rapeseed seedlings to a certain extent.

Regarding important antioxidant enzymes, SOD, POD, and CAT can remove a large amount of accumulated ROS under adversity stress. Their synergistic effect can maintain the integrity of cell membrane structure, inhibit membrane lipid peroxidation, and alleviate the oxidative damage suffered by plants [46]. Our current findings found that the low-temperature environment induced a significant increase in SOD and POD activity. The application of wood vinegar at low temperatures continued to improve the activity of the SOD enzyme, while the activity of POD and CAT decreased to the same level as the normal temperature control (Figure 4). This result shows that rapeseed seedlings, after applying wood vinegar at a low temperature, mainly convert ROS generated under low-temperature stress into H_2O_2 by increasing SOD activity, as well as through the significant increase in proline content. Acidic substances can stimulate rapeseed seedlings to initiate the defense mechanism and cause an increase in seedling antioxidant enzyme activity [47]. A suitable concentration of wood vinegar can make these substances have a good synergistic effect in different aspects and improve the comprehensive resistance of the plant.

5. Conclusions

The results showed that low-temperature stress negatively affected the leaf area, total biomass, stomatal density, and water use efficiency. Under low-temperature stress, rapeseed leaves sprayed with wood vinegar mainly enhance SOD enzyme activity, reduce leaf malondialdehyde content, and alleviate oxidative damage caused by low temperature, increasing proline, soluble protein, and soluble sugar content to raise the osmotic adjustment ability of the plant. The application of wood vinegar under low-temperature stress improved the stomatal density, water use efficiency, photosynthesis, and biomass accumulation of rapeseed, and thus improved the resistance of rapeseed to low-temperature stress. The current study shows that spraying wood vinegar on rapeseed can improve farmers' income, and spraying wood vinegar twice on rapeseed seedlings suffering from low-temperature could alleviate the low-temperature damage. In addition, wood vinegar is a kind of biomass clean energy material with low cost and high benefit. It has broad application prospects and can be widely applied in rapeseed fields.

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