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MICROCAPSULE SUSTAINED-RELEASE TECHNIQUE CAN PROLONG THE EFFECT OF JASMONATES ON NEEDLES OF *LARIX KAEMPFERI* (LAMB.) CARRIERE

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Abstract

The controlled release of pesticide microcapsules is an innovative approach that extends the duration of effectiveness, minimizes the loss of the active substance, and lessens the environmental footprint, thus supporting sustainable practices. Jasmonic acid (JA) and its methyl ester derivative, Methyl jasmonate (MJA), are recognized inducers of plant resistance. They have been reported to markedly elevate the levels of critical defensive proteins in *Larix kaempferi* seedlings upon application. However, the high volatility and fleeting efficacy of these compounds pose challenges. This research utilized chitosan and Tween 20 to engineer sustained-release microcapsule suspensions containing JA and MJA, aiming to compare the inducement effects pre- and post-gradual release. The findings revealed that both treatments significantly bolstered the defense protease concentrations in the needles. While the JA and MJA slow-release treatment induction rate was slower within the initial 1-3 days, at the later stages induction levels surpassed those of direct spraying to some extent. Moreover, it extended the effective period of JA and MJA by 5-10 days. Specifically, the JA sustained-release treatment increased the effective induction period for PAL, PPO, CAT, and POD by 5 days, whereas the MJA treatment did the same for CAT, POD, and PAL but did not extend PPO's efficacy duration. These outcomes demonstrate that the JA and MJA sustained-release microcapsule suspensions can enhance the induction effects of these compounds, offering theoretical insights for the practical application of JA and MJA induced resistance techniques in forestry.

Key words: Microcapsule sustained-release technology, Jasmonic acid, Methyl Jasmonate, Defense protein, *Lafix kaempferi*

Introduction

Induced insect resistance is a plant's defensive reaction to external stressors such as physical damage, herbivory, and exposure to exogenous substances. When subjected to these stressors, plants can alter herbivore behavior or reduce their attraction by synthesizing toxic secondary metabolites, defensive proteins, repellents, modifying their nutritional content, or releasing volatile organic compounds that lure natural predators. This multifaceted response serves to shield the plants from further damage or prevent attacks, thereby fulfilling the objective of insect deterrence and protection (Hammerschmidt & Nicholson, 1999, Hammerschmidt, 2007; Eyles *et al*., 2010, Fürstenberg-Hägg *et al*., 2013). Current research has identified numerous biotic and abiotic elements that can elicit a plant's innate defensive mechanisms. Induced resistance has been extensively studied within various horticultural and agricultural frameworks, and its practical deployment has been confirmed as effective (Alborn *et al*., 1997, Agrawal *et al*., 1999, Vallad & Goodman, 2004, Walters, 2009).

Jasmonic acid (JA) and its derivative, Methyl jasmonate (MJA), are widely recognized compounds involved in the study of plant-induced resistance (Creelman & Mullet, 1995, Glauser *et al*., 2008, Jiang & Yan, 2018). As crucial signaling molecules in plant biology, JA and MJA have been implicated in the regulation of a variety of defense gene expressions, responses to physical injury and disease, and they also play a significant role in plant growth, development, and stress response (Fonseca *et al*., 2009, Yu *et al*., 2019). Research indicates that JA content in plants surge rapidly following feeding by herbivores, pathogen infection, or mechanical damage. Subsequently, the expression of plant-specific genes is triggered, leading to the production of jasmonate-induced proteins (JIPs) that initiate the plant's induced defense response, thereby facilitating disease and pest resistance (Kazan & Manners, 2008, Wu *et al*., 2008, Moreira *et al*., 2009). *Larix kaempferi* (Lamb.) Carrière, a deciduous species of the family Pinaceae, is valued for its rapid growth and high-quality wood. Its introduction and cultivation have substantially enhanced domestic timber resources, contributed significantly to urban greening, and possessed considerable ecological benefits. Employing the concept of induced resistance, JA and MJA have been directly applied to larch seedlings in preliminary studies. These applications have successfully increased the levels of vital defense proteins in the seedlings (Peng *et al*., 2019). However, the induction effect of direct spraying can only be maintained for 5 days. The high volatility, rapid diffusion, and transient effectiveness of these compounds limit their large-scale application in forestry contexts. Notably, up to 90% of traditional agricultural pesticides are either lost or broken down in the environment, failing to deliver effective pest control, which in turn escalates costs and environmental contamination (Berg *et al*., 1999, Igbedioh, 2010). Microcapsule-based controlled release technology offers a solution to these issues, representing an established strategy for the sustained release of substances such as drugs, enzymes, vitamins, pesticides, fragrances, and catalysts, thus playing a significant role in various fields (Dubey *et al*., 2009, Zhao *et al*., 2019).

Micro capsule based controlled release technique is a well-established technique of surface modification and encapsulation. It involves coating dispersed solids, liquids, or gases within inorganic or organic polymer membrane materials (wall materials), thus forming minute particles (Dubey *et al*., 2009, Zhao *et al*., 2019). The resulting small particles range in size from nanometers (nm) to micrometers

(μm) to millimeters (mm), and are referred to as nanocapsules, microcapsules, particles, and microspheres, respectively. The encapsulated material is known as the core or capsule core, while the film-forming material is termed the wall material. Because the capsule wall possesses numerous micropores, it has good semi-permeability. Liquids or water-soluble substances within the microcapsules can be released through the wall by dissolution, osmosis, or diffusion (Lin *et al*., 2014). For instance, microencapsulated DEET showed significantly higher repellent activity in the hindgut of *Aedes aegypti* and *Anopheles aegypti* mosquitoes compared to the crude oil form of DEET (Rutledge *et al*., 1996). Miao (1982) utilized methyl parathion microcapsules to control older larvae of larch (7-8 years) and younger larvae (3-4 years), achieving improved results over emulsifiable concentrates with both rapid action and a lasting effect, allowing for substantial dosage reductions. This method is particularly well-suited for consistent, low-volume application both on the ground and aerially.

Therefore, in this study, we used *L. kaempferi* as the subject and compared the activity differences of crucial resistance enzymes between JA and MJA in the form of slow-release microcapsule suspensions and direct spraying of the compound. Our goal was to enhance the technology of slow-release microcapsule induction in *L. kaempferi* and provide theoretical guidance for research on the induced resistance of this species.

Material and Methods

Plant materials: The experimental site is situated in the seedling breeding center of Changlinggang Forest Farm, located in Jianshi County, Enshi Prefecture, China, at coordinates 30°48' N, 110°03' E. The site lies at an elevation of 1,600 to 1,900 meters and is characterized by a north subtropical climate. This forest farm hosts extensive plantations of *L. kaempferi*, serving not only as a national tree seed base but also as the largest *L. kaempferi* cultivation area in southern China. In early May 2020, 1,000 healthy 2-year-old *L. kaempferi* seedlings were selected from the experimental nursery. These were cultivated under natural conditions with strict management to prevent plant diseases, insect pests, and man-made mechanical damage, ensuring their suitability for future research.

Preparation of compounds and sustained-release capsules: Building upon previous research findings (Peng *et al*., 2019) and adopting the methodology of Lin *et al*., (2014), the optimal dosage for each strain of JA and MJA was set at 5 ml. The concentration for direct spraying was determined to be 1 $\mu L \cdot L^{-1}$.

For the sustained-release capsule suspension, the core-to-wall ratio was set to 3:1 (the core-to-wall ratio is the proportion of the core material to the wall material). Based on previous experience (Lin *et al*., 2014), JA and MJA microcapsules were produced utilizing chitosan as the wall material and maintaining a core-to-wall ratio of 3:1. First, 5 g of chitosan (Sinopharm Chemical Reagent Co., Ltd) was precisely measured and placed into a three-necked round-bottom flask; then, 135 mL of deionized water and 15 mL of glacial acetic acid (10 %) (Shanghai Macklin Biochemical Technology Co., Ltd) were added into the solution and stirred at a low speed for 30 min. 15 g of core material (JA or MJA, Shanghai Macklin Biochemical Technology Co., Ltd) was weighed, introduced into the flask, and emulsified with a mixture of 0.15 g of Tween 80 and 0.15 g of Tween 20 (Sinopharm Chemical Reagent Co., Ltd). The pH of the suspension was adjusted to 3 using glacial acetic acid, followed by the addition of 20 mL sodium sulfate solution (10 %) for flocculation. The temperature of the water bath was set at 10°C, added 140 mL of deionized water to the system, adjusted the pH value of the system to 9 with a 10% sodium hydroxide solution, added 12 mL of glutaraldehyde for cross-linking, and stir at low speed for 1 hour. Then, the system was heated to room temperature, the solution in the three-necked round-bottom flask was poured into a funnel and filtered using a vacuum pump. After washing, the microcapsules were collected and placed into a wide-mouth bottle, sealed for later use.

Preparation of microcapsule suspension: To prepare the microcapsule suspension, Tween 20, calcium chloride as a dispersant, and sodium carboxymethyl cellulose as an anti-settling agent were used. The resulting microcapsules were processed into a microcapsule suspension

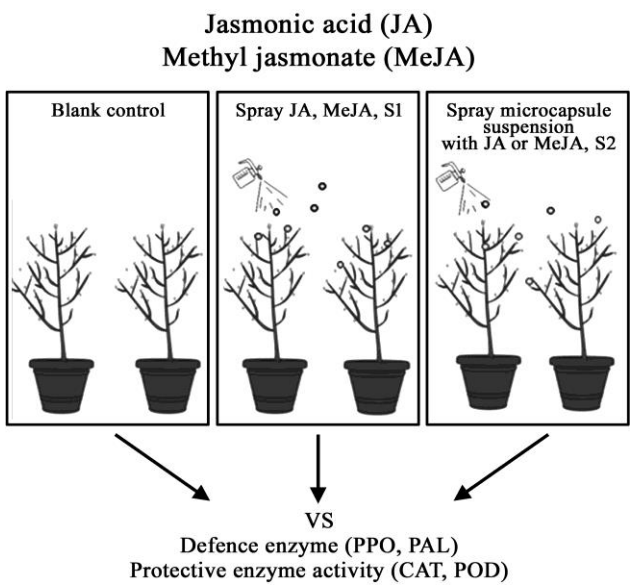

Fig. 1. Treatment of *Larix kaempferi* with exogenous JA and MJA.

Seedling treatment: At the end of July, robust *L. kaempferi* seedlings were divided into seven groups, with each group comprising 45 seedlings. As depicted in Fig. 1, treatments with JA and MeJA compounds, as well as their respective slow-release capsule suspending agents, were administered to the seedlings on a sunny morning, under conditions of 24 ± 1°C temperature and 70 ± 10% relative humidity using a spray bottle. A solvent of the same volume served as the control. The details of the spraying schedule are provided in Table 1. Needles from the healthy branches of each group were collected on the 1st, 3rd, 5th, 10th, 15th, 20th, and 25th day following treatment. For sampling, needles from five seedlings of the same treatment or control group were pooled to form one sample, with three such samples taken from each group. The samples were labeled as JA, MJA, S1, JA (SR), MJA (SR), S2, and CK, respectively. All samples were stored at -40°C in a freezer until further testing and analysis.

Table 1. Treatment of *L. kaempferi* with exogenous JA and MJA.

Groups	Inducer	dose/ (mL)	Concentration (uL. L^{-1})/ Ratio of Core to Wall	Sample code
1	JA	5	1	JA
2	MJA	5	1	MJA
3	Solvent 1 (water)	5	0	S1
4	Microcapsule suspension with JA	5	3-1	JA (SR)
5	Microcapsule suspension with MJA	5	3-1	MJA (SR)
6	Solvent 2 (Microcapsule suspension)	5	0-1	S2
7	Blank control	5	0	CK

Assays of enzyme activity: An appropriate quantity of fresh *L. kaempferi* samples was ground in liquid nitrogen. Subsequently, 0.5 g of the ground samples was precisely weighed and preserved at low temperature. The enzyme activities of phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), catalase (CAT), and peroxidase (POD) were measured using assay kits obtained from Suzhou Keming Biotechnology Co., LTD. PAL and PPO (defense enzymes), CAT and POD (protective enzyme) are integral to plant defense and are commonly utilized as indicators of resistance levels (Khattab & Khattab, 2005, Heidari, 2009, Heredia & Cisneros-Zevallos, 2009, Gould *et al*., 2009, He *et al*., 2011). Detailed experimental procedures were conducted according to the guidelines provided in the enzyme activity assay kit instructions.

Data analysis: SPSS 24.0 was used to analyze the variance of the experimental data, and LSD method and Duncan methods were used to test the significance of the difference.

Results

Effects of JA, MeJA volatile and the corresponding slow-release microcapsule suspension on the protective enzymes activity of ***L. kaempferi*** n**eedles:** As depicted in Fig. 2, following the application of JA, there was a significant increase in CAT enzyme activity within the first 1-5 days, peaking on the 5th day. The activity of CAT then declined from the 5th day, with no discernible difference between the JA-treated and control groups on the 20th day, indicating an induction effect lasting over 15 days. With the slow-release JA treatment, CAT enzyme activity showed no significant difference from the control (CK) on the first day but increased significantly between the 3rd and 10th days, reaching a maximum on the 10th day. This activity was notably higher than that of the control on both the 15th and 20th days, with no difference observed on the 25th day. The induction effect from the slow-release treatment persisted for over 20 days and was more effective than the spray treatment starting from the 10th day.

The activity of the POD enzyme exhibited a similar trend. Following JA spraying, POD activity rose significantly within the initial 1-3 days, then started to decline after the 3rd day. After 25 days, there was no difference between the treatment and control groups, and the induction effect lasted for over 20 days. With slow-release treatment, POD activity increased considerably on the third day and remained stable, showing a decline after the 15th day. However, the activity was still significantly higher than that of the control at 25 days, with the induction effect enduring for over 25 days. The efficacy of the slow-release treatment from the 10th day outperformed the spray treatment.

As shown in Fig. 3, after the application of MJA, the activity of the CAT enzyme significantly increased within the first 1-5 days, peaked at 5 days, and then exhibited a downward trend. There was no significant difference in enzyme activity compared to the control group on the 20th day, indicating that the induction effect lasted for more than 15 days. After the sustained-release treatment with MJA, CAT enzyme activity did not differ significantly from the control (CK) on the first day but increased significantly from the 3rd to the 10th day, reaching the peak on the 10th day. By the 25th day, there was no significant difference between MJA-treated and control groups, and the induction effect extended over 20 days. The slow-release treatment showed better efficacy than the spraying method starting from the 15th day.

Following the spraying of MJA, POD enzyme activity surged on the first day and then significantly decreased by the 5th day. No difference between the treatment and control groups was observed on the 20th day, and the induction effect lasted for more than 15 days. After the slow-release MJA treatment, the enzyme activity was increased significantly within the first 1-3 days and then declined after 3 days, with effective activity persisting until the 20th day. The slow-release treatment showed greater efficacy than the spray treatment from the 10th day onward.

Effects of JA, MeJA volatile and the corresponding slow-release microcapsule suspension on the activity of defense enzymes in *L. kaempferi* needles: As depicted in Fig. 4, following the application of JA, PPO enzyme activity was increased significantly on the first day, with a slight increase noted on the fifth day before exhibiting a downward trend. By the 25th day, there was no significant difference in PPO activity between the JA-treated and control (CK) groups, and the induction effect was observed to last for more than 20 days. In the JA slow-release treatment group.

PPO activity increased significantly on the first day and began to decline after the 15th day. However, the overall induction effect was higher than that of the control group, lasting for more than 25 days. Starting from the 10th day, the induced effect of the slow-release treatment was better than that of the spray treatment.

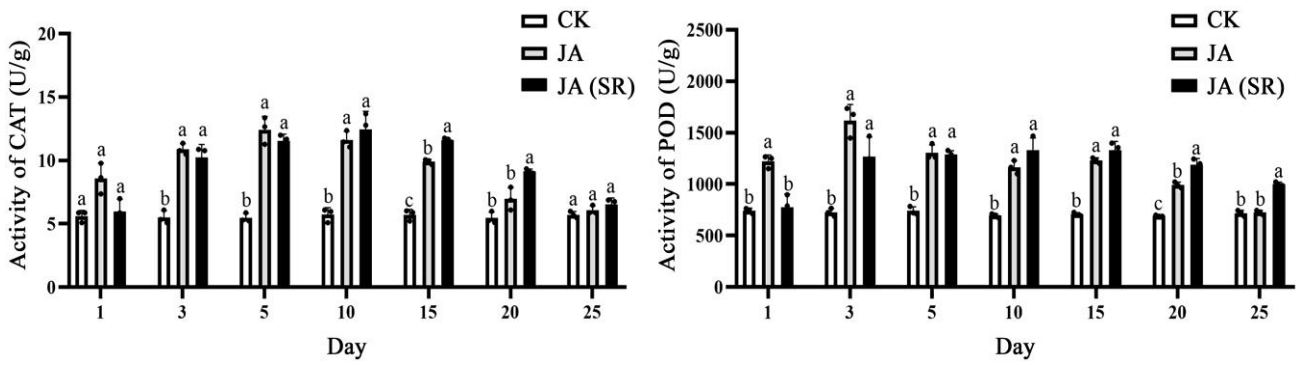

Fig. 2. Effects of JA volatile and the corresponding slow-release microcapsule suspension on the protective enzyme activity of *Larix kaempferi* needles. The values presented in the figure are means ± standard deviations (N = 3). Different lowercase letters represent significant differences between different treatments in the same column ($p<0.05$). (The four pictures below are the same as this one).

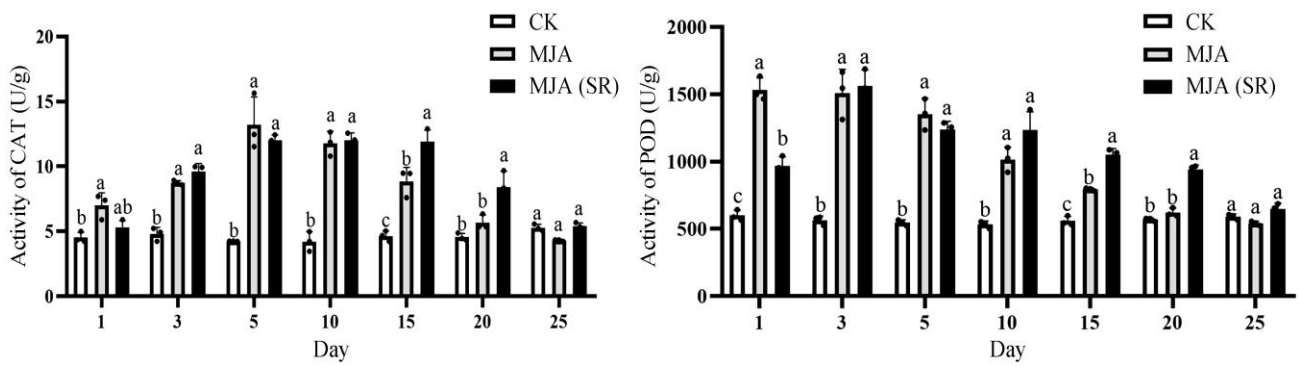

Fig. 3. Effects of MJA volatile and corresponding slow-release microcapsule suspension on the protective enzyme activity *Larix kaempferi* needles.

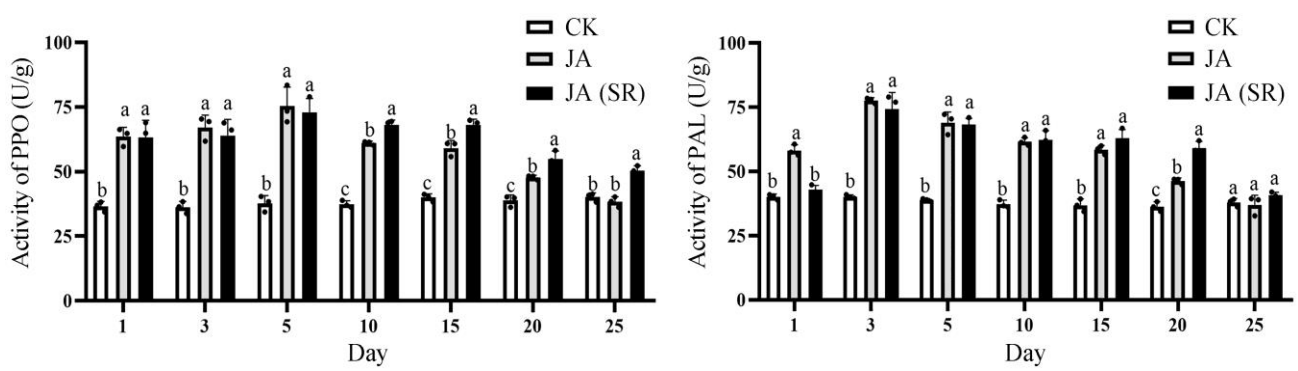

Fig. 4. Effects of JA volatile and the corresponding slow-release microcapsule suspension on the defense enzyme activity of *Larix kaempferi* needles.

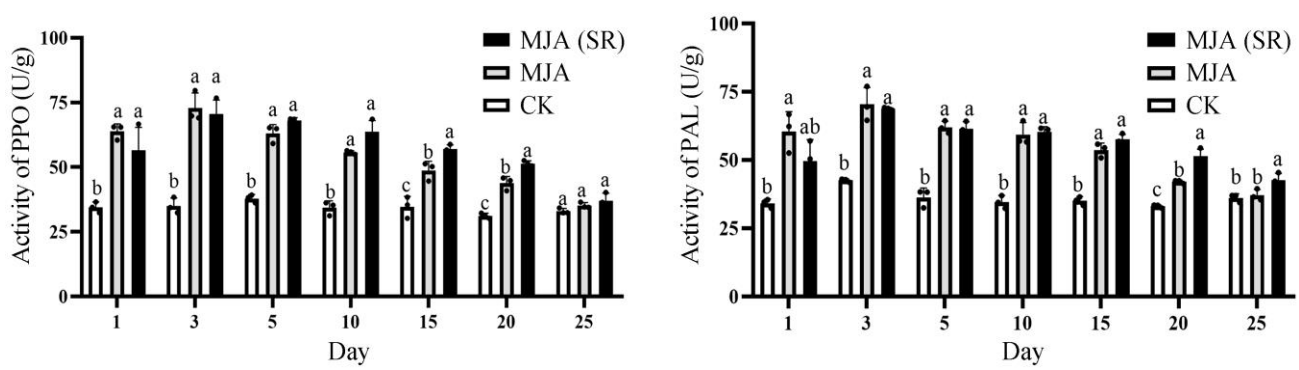

Fig. 5. Effects of MJA volatile and corresponding slow-release microcapsule suspension on the defense enzymes activity of *Larix kaempferi* needles.

PAL enzyme activity increased significantly within the first 1-3 days after JA application and then slowly decreased after the third day. At 25 days, there was no difference in PAL activity between the CK and the treatment groups, with the induction effect lasting over 20 days. In the slow-release treatment group, PAL activity did not differ significantly from CK on the first day but increased significantly by the third day. Afterward, a downward trend was observed, leveling off with the control group at 25 days, thus indicating an induction effect extending over 20 days. Post 15 days, the effect of slow-release induction surpassed that of the control group.

Fig. 5 illustrates that after the application of MJA, PPO enzyme activity showed a significant increase during days 1 to 3, followed by a decrease thereafter. By day 25, no significant difference was observed in PPO enzyme activity between the control group (CK) and the treatment group, with the induction effect lasting for over 20 days. Although the trend in PPO enzyme activity in the MJA slow-release treatment group mirrored that of the spray treatment, the induction effect of the slow-release was somewhat greater from day 5 onwards.

For PAL enzyme activity, a significant increase was noted within the first 1 to 3 days after MJA application, which then slowly declined after day 3. At day 25, PAL enzyme activity was not significantly different from the control group, and the induction effect was sustained for over 20 days. In the slow-release treatment group, PAL enzyme activity was significantly increased within the initial 1 to 3 days and then decreased after day 3. The overall induction effect was higher compared to the control group, extending over 25 days. Starting from the 10th day, the slow-release treatment's induced effect was superior to that of the spray treatment.

Discussion

Induced resistance in forests plays a crucial role in pest management due to its adaptability and flexibility. Compared to the inherent resistance mechanisms of plants, induced resistance against pests is considered to be more cost-effective and energy-efficient, imposes a lower metabolic burden on the plants, and demonstrates species specificity (Fürstenberg-Hägg *et al*., 2013). Studies have shown that the direct application of JA and MJA can lead to an initial increase and subsequent decrease in the activities of key resistance enzymes in *L. kaempferi*, but this effect is short-lived, highlighting the need for improved methods for prolonged application (Peng *et al*., 2019). Therefore, building on previous research, this study examined the differences between sustained-release treatment and direct spraying of JA and MJA in the induction of resistance, utilizing microencapsulation technology for sustained release.

The results indicated that both treatments significantly enhanced the activities of CAT, POD, PAL, and PPO throughout the plant, with an initial increase followed by a decrease, consistent with previous findings (Peng *et al*., 2019). These enzymes are crucial for the inducible resistance of plants. CAT and POD are integral in the defense mechanism against oxidative stress, serving as vital protective enzymes (Khattab & Khattab, 2005, Heidari, 2009, He *et al*., 2011). The activity of PAL and PPO contributes to the plant defense system by catalyzing the production of secondary metabolites (Heredia & Cisneros-Zevallos, 2009, Gould *et al*., 2009). Both direct spraying and slow-release methods increased the levels of these defense proteins, suggesting that either approach can activate the systemic defense response of the plant.

However, the slow-release treatment exhibited a significantly stronger induction effect than the direct spraying method. Although the JA and MJA slow-release treatment had a slower initial induction speed during the first 1-3 days, the induction levels at later stages were higher compared to direct spraying, and the treatment could extend the effective duration of the compounds to a certain degree. The slow-release treatment of JA prolonged the effective induction period of CAT, POD, PAL, and PPO by 5 days. After 10 days, the activities of CAT, POD, and PPO in the slow-release treatment were higher to a certain extent than those in the direct spray treatment, and the activity of PAL was higher after 15 days. The effects of MJA slow-release treatment differed from those of JA, extending the effective induction period of CAT, POD, and PAL by 5 days; however, it did not prolong the action time of PPO. After 5 days of MJA slow-release treatment, PPO activity exceeded that of direct spraying. After 10 days, the activities of POD and PAL were higher, and after 15 days, CAT activity surpassed that of direct spraying. We think that this may be attributed to the slow-release microcapsules' ability to reduce the volatility and degradation rates of the compounds, while their strong adhesiveness also increases their retention time on the foliage. Microcapsules have been demonstrated to significantly improve the efficacy of treatments on plants. For instance, abamectin encapsulated in sticky polydopamine (PDA) microcapsules showed increased retention time on leaf surfaces, and the PDA shell provided effective protection against ultraviolet radiation, safeguarding abamectin from photodegradation (Jia *et al*., 2014). In line with the sustained release effects observed with abamectin, microcapsules of Cypermethrin/polyurea, PHB fluralin (based on pyrethroid herbicides), and capsaicin coated with chitosan and carboxymethyl chitosan all exhibit improved stability, adhesiveness, and drug efficacy (Kamble *et al*., 2018, Cao *et al*., 2019; Cui *et al*., 2022). Corresponding to these findings, our slow-release microcapsules displayed significantly enhanced induction time and efficiency when compared to direct spray treatments, underscoring the efficacy of slow-release microcapsules in promoting resistance in *L. kaempferi* to JA and MJA.

Conclusions

Induced resistance, as a vital component of green pest control strategies, can significantly reduce pesticide use. The forest environment is dynamic and complex, which often limits the effectiveness of directly sprayed resistance inducers. However, the implementation of slow-release technology can effectively extend the duration of the agent's activity, providing important insights for the practical application of resistance inducers. Exogenous jasmonic acid compounds have been shown to enhance the activity of defense proteins in *L. kaempferi* and boost its resistance to insects. Slow-release treatments can effectively increase this induced effect, which holds substantial value for sustainable pest management. Further investigations should focus on assessing the actual insect-resistant effects of the treatment, exploring the dynamics of volatile substances and enzyme activity in the needles, and evaluating the cost-effectiveness of

practical applications in forestry. Such research will contribute to the advancement of JA and MJA induced resistance in the integrated management of forest pests.

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