EFFECTS OF INJECTION OF ETHREL, METHYL JASMONATE, AND SALICYLATES AND RAFFAELEA QUERCIVORA INOCULATION ON SAPWOOD DISCOLORATION IN QUERCUS SERRATA

Boontida Moungsrimuangdee¹, Hiroyuki Moriwaki¹, Masanori Nakayama², Shintaro Nishigaki³ and Fukuju Yamamoto²*

SUMMARY

Ethrel (Et), methyl jasmonate (MJ), methyl salicylate (MS), sodium salicylate (NS), and mixed combinations of these chemicals were horizontally injected into stems to induce defense responses in Quercus serrata Thunb. Four months after wounding with the application of those chemicals, the extent of sapwood discoloration was observed in tangential and axial directions. The combination of MJ and Et (MJ+Et) induced the greatest discoloration among all treatments. Sodium salicylate (NS) or methyl salicylate (MS) alone increased the discolored area to a lesser degree than did MJ, but defensive responses were obviously more accelerated when the former were added to the latter in the combination treatments. In particular, induced discoloration was noticeably achieved following MJ or Et combined with NS rather than as individual treatments. In contrast, neither salicylate appeared to promote discoloration when combined with the MJ+Et treatment. Wounds challenged with an inoculation by a bark beetle vectored fungus, Raffaelea quercivora, developed significantly greater sapwood discoloration than did non-pathogen inoculation, in all directions.

Key words: Ethylene, jasmonic acid, Quercus serrata, Raffaelea quercivora, salicylic acid, sapwood discoloration.

INTRODUCTION

Defense responses in plant cells against herbivory and pathogen invasions or mechanical wounding consist mainly of necrosis and formation of defensive substances accumulated in specialized or non-specialized compartments surrounding the wounded zone, activated as a protective barrier to cease attacks following infection. In living trees, the process is well described by a previously developed compartmentalization concept, specifically a model named compartmentalization of decay in trees (CODIT) in which trees are considered as highly compartmented organisms, and discolored and

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decayed wood associated with trunk wounds are compartmentalized (Shigo & Marx 1977). In other words, sapwood actively responds to wounding and/or infection by the formation of structural and chemical boundaries that limit the affected tissue to a minimal volume. These boundaries represent compartmentalization walls that prevent the further spread of decay throughout the wood and ensure the survival of the tree (Shortle et al. 1996).

Ethylene has an important function in the modification of growth and morphology of stems of woody species (Savidge 1988). It has been demonstrated that ethylene production by stems increases as a result of various stresses including flooding (Yamamoto & Kozlowski 1987; Yamamoto et al. 1995), gravity (Du et al. 2004), insect and pathogen attack (Shain & Hillis 1972; Popp et al. 1995; González & Campos 1996), application of chemicals (Hudgins & Franceschi 2004), and wounding (Yamanaka 1985). In conifers, such ethylene evolution under the induced defense reaction is related to a formation of traumatic resin ducts (Hudgins et al. 2006), oleoresin production (Wolter & Zinkel 1984), and the accumulation of phenolic compounds (Shain & Hillis 1973).

Methyl jasmonate has been found to induce anatomical defense reactions in conifer stems such as cell swelling due to an increase of phenolic content in polyphenolic parenchyma cells and formation of additional polyphenolic parenchyma cells and of traumatic resin ducts in the cambial zone (Franceschi et al. 2002). Application of methyl jasmonate also has been shown to protect conifer seedlings against damping-off disease (Kozlowski et al. 1999) and pine weevils (Heijari et al. 2005). A link between methyl jasmonate and ethylene has been reported in many plants such as tomato, tobacco, and Arabidopsis (Saniewski et al. 1987; Xu et al. 1994; Penninckx et al. 1998). These findings show that plant defense genes are synergistically activated by ethylene and methyl jasmonate. Methyl jasmonate applied to stem surfaces has been shown to induce higher ethylene production than does wounding in conifers (Hudgins & Franceschi 2004).

Salicylic acid is another chemical compound often used to activate defense mechanisms, and it has been shown to be involved in resistance-signaling pathways. Exogenously supplied salicylic acid stimulates pathogenesis-related gene expression and disease resistance in a wide variety of plant species (Pieterse & van Loon 1999; Sudha & Ravishankar 2003). An increase in salicylic acid has been shown in Norway spruce exposure to methyl jasmonate, indicating a significant role of methyl jasmonate in induced salicylic acid accumulation in conifers (Kozlowski et al. 1999). In addition, ethylene, jasmonic acid, and salicylic acid are the essential components involved in the establishment of plant disease resistance (Nürnberger & Scheel 2001).

Although a number of studies have demonstrated the effects of those compounds on stem defense induction (Kozlowski et al. 1999; Franceschi et al. 2002; Hudgins & Franceschi 2004), no published information exists to date on the role of the potent hormone-induced resistant mechanism in Quercus species. Such information is needed to combat the large-scale Japanese oak wilt caused by bark beetle (Platypus quercivorus) infection by the vectored fungi Raffaelea quercivora, which has occurred throughout Japan since the 1980s (Urano 2000). The purpose of this study was to investigate the effects of exogenous application of potent chemical elicitors including ethylene, methyl jasmonate, and salicylic acid in defense responses involving discolored wood
of *Quercus serrata*. The discoloration of sapwood as a part of physiological defense reactions may differ in individual and combination applications of these compounds. Additionally, discoloration response to the pathogenic fungus *R. quercivora* inoculation was investigated in this study to provide insight into defense reactions following a pathological infection.

**MATERIALS AND METHODS**

*Ethrel, methyl jasmonate, sodium salicylate and methyl salicylate treatments*

Healthy *Quercus serrata* trees growing in a natural stand in the Tottori University Forests, Hiruzen, Okayama Prefecture, Japan, were selected for this study. Ethrel (Et), an

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ethylene releasing compound, methyl jasmonate (MJ), methyl salicylate (MS), sodium salicylate (NS), and combinations of these (1% concentration for each compound) were emulsified in lanolin plus Tween 80 as a paste material. Lanolin with Tween 80 was prepared as the control. Two trees in each treatment were applied with the various components mentioned above (Table 1). On 6 July 2008, three holes (5 cm in depth, 4 mm in diameter) were pierced on opposite sides in the same plane of the trunk at three different heights (60, 140 and 220 cm) above ground level to avoid overlap of each wound using a hand drill (18 replications per treatment) (Fig. 1). Then the paste was injected into drilled holes using a 20-ml syringe and covered with gummed tape.

**Raffaelea quercivora isolation and inoculation**

Raffaelea quercivora obtained in 1995 at Torigoe in Iwami town, Tottori Prefecture, Japan, was grown on wooden toothpicks placed on potato dextrose agar media and incubated in the dark at 25 °C for 5 weeks prior to inoculation. The inoculation was carried out on 14 and 15 July 2008. Inoculated toothpicks were inserted into holes (5 cm in depth, 3 mm in diameter) drilled in two trees, and sterilized wooden toothpicks were inserted into stems of two other trees as a control (Table 1). All inoculation holes were sealed with sterilized absorbent cotton containing water and with Parafilm and gummed tape.

**Analysis of sapwood discoloration**

Trees treated with Ethrel, methyl jasmonate, sodium salicylate, methyl salicylate, and mixed combinations of these chemicals, were harvested on 1 November, and Raffaelea...
**RESULTS**

**Tangential sapwood discoloration**

Application of NS to *Quercus serrata* appeared to induce tangential discoloration to a greater degree than did MS. In contrast, NS increased the discoloration to a lesser degree than did MS when in combination with other components. A combination of MJ and MS treatment (MJ+MS) was the most effective in the induction of discoloration in the tangential direction. However, no significant difference was observed compared to the combined MJ and NS treatment (MJ+NS) (Fig. 3).

The extent of discoloration with a combination of Et and NS (Et+NS) or MS (Et+MS) showed no significant difference, and no difference was observed among the Et treatments. The combination of MJ and Et (MJ+Et) increased the discoloration to a greater extent than did individual MJ or Et treatments (Fig. 3).

*Raffaelea quercivora* inoculation in *Q. serrata* showed induction of sapwood discoloration with relation to defense responses against pathogen invasion in our study. This induction was found at a proximal rate to the control treatments (lanolin + Tween 80). However, the discolored lengths differed significantly between *R. quercivora* inoculation and the control inoculation (Fig. 3).

**Axial sapwood discoloration**

Greater axial discoloration lengths were observed in the upper than in the lower part of the trunk stimulated with the various elicitors, except in the non-*R. quercivora* treatment. In particular, in the combined MJ+Et treatment, the upper discolored length was significantly longer than in the lower part (Fig. 4).
Figure 3. Tangential sapwood discoloration length of *Quercus serrata* four months after wounding through the application of chemicals and with fungal inoculation. – Et: ethrel; MJ: methyl jasmonate; MS: methyl salicylate; NS: sodium salicylate. – Data are represented as mean and ± SE of 18 replications for each treatment. – Different letters in each column indicate significant differences (P < 0.05) using Scheffe’s test. – ** = difference significant at P < 0.01.

Figure 4. Axial sapwood discoloration length of *Quercus serrata* four months after wounding through the application of chemicals and with fungal inoculation in upper and lower parts of the treated wound. – Et: ethrel; MJ: methyl jasmonate; MS: methyl salicylate; NS: sodium salicylate. – Data are represented as mean and ± SE of 18 replications for each treatment. – Different letters in each column indicate significant differences (P < 0.05) using Scheffe’s test. – ** = difference significant at P < 0.01.
Generally, the axial discoloration lengths in NS or MS combination treatments appeared to be longer than those in treatments excluding NS or MS. These phenomena were exceptional with the addition of NS or MS to MJ+Et. Moreover, the discolored length in the MJ+Et application was the longest among the treatments, whereas addition of NS or MS resulted in shorter lengths. NS showed the greatest stimulation of discoloration in *Q. serrata* when combined with Et (Et+NS), whereas discoloration in MS treatments was longest in combination with MJ (MJ+MS). In contrast, MS was the least effective in combination with Et (Et+MS) (Fig. 4). Acceleration of axial discoloration in *Q. serrata* was significantly greater with *R. quercivora* than with the sterilized inoculations in both the upper and lower parts (Fig. 4).

**Area of sapwood discoloration**

In both the upper and lower parts, combined treatments of NS or MS seemed to develop areas of discoloration in treated *Q. serrata* to a greater degree than those treatments without those substances, except in the combination of MJ and Et (MJ+Et). In addition, maximum induction was found in the upper part of the induced wounds in the MJ+Et treatment (Fig. 5). Similarly, NS and MS appeared to not promote discoloration when combined with the MJ+Et treatment, as shown in the axial section.

NS or MS alone enhanced the discolored area less than did MJ, but defensive responses obviously were more accelerated with the former than with the latter in the combination treatments. In particular, induced discoloration was noticeably achieved following addition of NS to MJ or Et as a combination rather than as individual treatments (Fig. 5).

![Figure 5](image-url)  
Figure 5. Area of sapwood discoloration of *Quercus serrata* four months after wounding through the application of chemicals and with fungal inoculation in upper and lower parts of the treated wound. – Et: ethrel; MJ: methyl jasmonate; MS: methyl salicylate; NS: sodium salicylate. – Data are represented as mean and ± SE of 18 replications for each treatment. – Different letters in each column indicate significant differences (P < 0.05) using Scheffe’s test. – ** = difference significant at P < 0.01.
The area of discoloration in the *R. quercivora* inoculation showed a similar pattern in the tangential and axial lengths and was significantly greater than in the sterile inoculation (Fig. 5).

**Histochemical observation**

Deposits were microscopically observed in the discolored sapwood of chemically treated and fungal-inoculated *Q. serrata*. Unstained radial sections showed yellowish-

Figure 6. Radial section showing accumulation of deposits in ray cells (arrows) of discolored *Quercus serrata* sapwood four months after wounding through the application of chemicals (A–E) and with *Raffaelea quercivora* inoculation (F). – A: Yellow to brown droplets without staining. – B: Total lipids stained black to deep blue with Sudan black B. – C: Lipids stained red with Sudan III. – D: Acidic lipids stained blue with Nile blue. – E & F: Phenolic compound stained black with ferric chloride and vessels filled with tyloses (arrowheads). – V: vessel. — Scale bars = 100 µm.
brown substances accumulated in ray cells (Fig. 6A). Additional staining with Sudan black B, Sudan III, Nile blue, and ferric chloride gave positive test results in all treatments. Lipids were stained black with Sudan black B, red with Sudan III, and blue with Nile blue (Fig. 6B, D). Phenolics were stained black with ferric chloride (Fig. 6E, F). Tyloses were detected in vessel lumens of discolored sapwood (Fig. 6E, F).

DISCUSSION

Discolored wood is formed in response to insect attack, wounding, microorganism colonization, and chemical elicitors (Shain 1995). The formation of discolored wood acts as a barrier zone for compartmentalization of infected trees to prevent the further spread of decay and pathogen invasion (Shigo & Marx 1977). Three subsequent major substances, including ethylene, jasmonic acid, and salicylic acid have been found to be important signaling compounds involved in plant defense mechanisms (Savidge 1988; Kozlowski et al. 1999; Nürnberger & Scheel 2001).

An increase in disease resistance by ethylene and ethylene production has been investigated among a diverse range of plant defense reactions against pathogenic fungal infection (Stahmann et al. 1966; Chappell et al. 1984). In sweet potato, tissue treated with ethylene prior to fungus (Ceratocystis fimbriata) inoculation showed an increase in resistance to black rot disease (Stahmann et al. 1966). In addition, their study also found that ethylene was detected in the infected tissue with C. fimbriata, but not above a culture of such fungi grown on potato dextrose agar, demonstrating that ethylene is an active volatile increasing resistance and enzymatic activity in the response of sweet potato to fungal inoculation. Chappell et al. (1984) reported that parsley (Petroselinum hortense) treated with an elicitor from the cell wall of the fungus Phytophthora megasperma caused rapid induction of ethylene biosynthesis in host cells. Infected stems of slash pine and loblolly pine with the bark beetle vectored fungi Ophiostoma spp. increased the rate of ethylene production, further indicating a greater rate of ethylene production associated with greater fungus virulence (Popp et al. 1995).

Methyl jasmonate has been found to be a part of the signaling mechanism inducing defense responses to insects and microorganisms in the conifer stem (Hudgins et al. 2003). The studies by Zeneli et al. (2006) and Krokene et al. (2008) have similarly reported that application of methyl jasmonate to Norway spruce enhanced resistance to the pathogenic bark beetle associate Ceratocystis polonica.

Our study results show that application of potent inducers of plant defense increases sapwood discoloration subjected to wound-induced responses in Quercus serrata. Furthermore, responsive discoloration increased remarkably when a combination of methyl jasmonate and Ethrel was used. This is supported by Xu et al. (1994), who have shown that defense genes accumulated in tobacco are significantly co-regulated by the combination of Ethaphon, an other ethylene releasing compound, and methyl jasmonate treatments. According to Hudgins and Franceschi (2004), application of methyl jasmonate in Norway spruce ultimately induces resistance mediated by ethylene, indicating methyl jasmonate and ethylene as key signaling compounds for inducible defense mechanisms.
A challenge with a combination of methyl jasmonate and methyl salicylate or sodium salicylate has been shown to activate enlargement of the discoloration to a greater degree than any of these treatments individually. This is supported by observations in tobacco, in which the combination of methyl jasmonate and salicylic acid had a strongly synergistic effect in the induction of related defense genes (Xu et al. 1994).

Ethylene in systemic acquired resistance is dependent on salicylic acid accumulation in Arabidopsis; low concentrations of salicylic acid with the addition of ethylene enhance the sensitivity of tissues to the action of salicylic acid (Lawton et al. 1994). In our study, only sodium salicylate had a desirable compatibility with Ethrel in discoloration induction, whereas methyl salicylate showed no response and reduced the barrier slightly compared to Ethrel alone. Likewise, adding both salicylates in combination with methyl jasmonate and Ethrel appeared to suppress the defense mechanism in Q. serrata. As a consequence, more information about the role of interaction signals in plant defense resistance in response to such compounds, as well as appropriate concentrations, should be considered and investigated.

In Japanese oak trees, infection with Raffaelea quercivora, whose vector is the ambrosia beetle Platypus quercivorus, results in the blockage of xylem sap ascent or loss of water conductivity, leading to the rapid wilting of host trees (Kuroda 2001; Kubono & Ito 2002; Murata et al. 2005). Xylem discoloration, as part of the defense response in Q. serrata to R. quercivora infection, is anatomically expressed by the secretion of deposits occluding fiber tracheids and small vessels following invasion (Kuroda 2001). In this investigation, inoculation of R. quercivora in Q. serrata significantly stimulated sapwood discoloration more than in the non-pathogen treatment. Furthermore, the development of discolored wood in response to R. quercivora inoculation of trees has been specifically examined by earlier researchers (Yamada et al. 2003; Murata et al. 2007).

Our study shows that sapwood discoloration in Q. serrata after induced wounding and artificial inoculation with R. quercivora apparently extends farther in the axial than in the radial or tangential wood alignment. Kuroda (2001) proposed that the area of discoloration following R. quercivora infection was wider in the longitudinal than in radial and tangential direction, reflecting the ability of fungal hyphae to elongate preferably along the vessel lumens rather than radially or tangentially through pits or the cell wall. These findings are similar to experiments on Eucalyptus globulus and E. nitens in which discoloration and decay caused by pruning, wounding, and white rot decay fungi inoculation was always greater axially than radially and/or tangentially (Deflorio et al. 2007).

In the application of chemical compounds, the absolute discolored length in the tangential direction slightly differs among all treatments compared to the axial direction. It is possible that internal transport of those compounds may be restricted tangentially through the thick walls and small pits in oak sapwood. Thus, a greater degree of discoloration in vertical direction indicates that chemical inducers are more actively transported longitudinally than tangentially.

Histochemical study of the occlusion in the discolored sapwood shows the presence of lipids and phenolic compounds in both the application of chemicals and fungus in-
occlusion. Similar compounds were observed in the reaction zone of *Quercus crispula* inoculated with *R. quercivora* (Yamada et al. 2003). In *Azadirachta indica*, phenolic compounds disappeared in artificially injured stems, and presence of phenolics in infected stems identified it as a protective compound against fungal infection (Rajput et al. 2009). However, biochemical variation in discolored wood is complex and involves a wide range of compounds associated with the metabolism of alkaloids by invading microorganisms (Hillis 1987). Histochemical changes in this study, therefore, generally demonstrate chemical defense traits exuded after induced wounding or fungal infection.

Finally, results from this study establish the roles of exogenous chemical elicitors that can enhance defense responses in oak stems. Further manipulation based on these fundamental data may improve our understanding of disease resistance against fungal infection in oak trees.

ACKNOWLEDGMENTS

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