Methionine-Riboflavin Mixtures with Surfactants and Metal Ions Reduce Powdery Mildew Infection in Strawberry Plants

Shiow Y. Wang

U.S. Department of Agriculture-Agricultural Research Service, Fruit Laboratory, Beltsville Agricultural Research Center, Beltsville, MD 20705

Dean Der-Syh Tzeng

Department of Plant Pathology, National Chung Hsing University, Taichung 40227, Taiwan, Republic of China

ADDITIONAL INDEX WORDS. *Fragaria xananassa*, biocidal agent, disease control, photodynamic effect

ABSTRACT. Foliar application of a mixture of methionine (1 mM) and riboflavin (26.6 μ M) reduced the severity of powdery mildew [*Sphaerotheca macularia* (Wallr. ex Fr.) Jacz. f. sp. *fragariae*] infection in 'Earliglow' strawberry (*Fragaria xananassa* Duch.) plants. Efficacy of this mixture on controlling powdery mildew infection was enhanced by supplements of copper, iron, and surfactants [sodium dodecyl sulfate (SDS), Triton X-100, Tween-20, or oxyalkylenemethylsiloxane (Silwet L-77)]. Free-radical scavengers (n-propyl gallate, thiourea) and antioxidants (α -tocopherol, β -carotene) reduced the efficacy of this mixture. Plants treated with a mixture of riboflavin (26.6 μ M), D,L-methionine (1 mM), copper sulfate pentahydrate (1 mM), and surfactants (SDS or Silwet L-77 at concentrations of 0.05% to 0.1%) showed a decrease in powdery mildew infection. Results of this study suggest that treatment with a mixture of methionine and riboflavin is beneficial to strawberry plants and may serve as an alternative to fungicides for controlling powdery mildew.

Powdery mildew [*Sphaerotheca macularia* (Wallr. ex Fr.) Jacz. f. sp. *fragariae*] is a serious fungal disease in strawberry (*Fragaria xananassa*) plants. Its control requires periodic application of fungicides. Because the use of chemicals poses potential harmful effects on the ecosystem, the application of various agricultural pesticides to control plant diseases has become an issue of great concern to the general public. Therefore, it is important to find an effective alternative method to control powdery mildew in strawberry plants and reduce dependency on fungicide use.

Nonconventional fungicide such as silicon (Si), has been shown to reduce the severity of powdery mildew in barley (Jiang et al., 1989), grape (Bowen et al., 1992), cucumber, muskmelon, zucchini squash (Menzies et al., 1992; Samuels et al., 1991), and strawberry (Wang and Galletta, 1996). Mixtures of methionine and riboflavin, in the presence of light, can generate various active oxygen radicals or active oxygen derivatives, including hydroxyl radical and singlet oxygen, which are strongly biocidal to a wide spectrum of microorganisms (Tzeng and DeVay, 1985, 1989; Tzeng et al., 1996a). A formulation containing this biocidal mixture as the main ingredient has been effective in controlling powdery mildew diseases in various crops (Tzeng, 1988; Tzeng et al., 1996b). The methionine and riboflavin mixture was also effective in inhibiting crown gall (*Agrobacterium tumefaciens*) infection of turnip, and controlling water-habituating pest organisms such as *Pythium* sp., larvae of *Culex* sp., *Chlorella* or *Chlamydomonas* sp., or even the notorious *Pomacea lineata* (Tzeng, 1988; Tzeng et al., 1987). The methionine and riboflavin mixture is toxic to *Phytophthora infestans* and can cause the

reduction of potato late blight disease (Jordan et al., 1992). *Verticillium dahliae*, *Peronophythora litchii*, *Botrytis cinerea*, and *Pseudomonas syringae* were all sensitive to the methionine and riboflavin mixture (Tzeng and DeVay, 1989). In view of all of the biocidal activities of the methionine and riboflavin mixture, this study was undertaken to determine the effectiveness of methionine and riboflavin mixture in combination with various metal ions and surfactants on controlling powdery mildew infection in strawberry plants. 'Earliglow' was used in this study because of its susceptibility to powdery mildew (Scott and Draper, 1975).

Materials and Methods

'Earliglow' strawberry plants propagated by runner cuttings at the six expanded leaves stage were used in this study. The plants were grown in pots (6.5 $\times$ 8.25-cm, E.C. Geiger, Inc., Harleysville, Pa.) containing Pro-Mix BX (Premier Brands Inc., Stamford, Conn.) in a greenhouse. Radiation sources in the greenhouse consisted of natural daylight and Watt-Miser incandescent lamps (Nela Park, Cleveland, Ohio), which provided a photosynthetically active radiation (PAR) level of ≈ 400 to 500 μ mol $\cdot$ m $^{-2}$ $\cdot$ s $^{-1}$ for 14 h $\cdot$ d $^{-1}$ (0600 to 2000 HR). Temperatures were set at 25 $^{\circ}$ C during the day and 20 $^{\circ}$ C at night. All plants were watered daily and fertilized biweekly with 150 mL/plant Peters nutrition solution (20N-20P-20K).

The methionine-riboflavin solution contained 1 mM L-methionine and 26.6 μ M riboflavin. This solution was supplemented with various metal ions, surfactants, free-radical scavenger and antioxidant compounds. Test solutions were prepared fresh, adjusted to pH 5.0 with 0.1 M HCl for each experiment, and applied under illumination (sunny day). Conidia of *S. macularis* f. sp. *fragariae* for the experiments were collected from infected strawberry leaves 1 h before inoculation by shaking mature colonies on infected strawberry leaves into a flask of 0.01% Tween-20 in distilled water. The number of conidia present was determined using a haemocytometer. As a pretreatment, powdery mildew

Received for publication 15 Apr. 1998. Accepted for publication 29 June 1998. We thank Kelly Y. Chang for technical assistance; Osi Specialties, Inc., Tarrytown, N.Y., for the sample of Silwet L-77. Use of a company or product name by the USDA does not imply approval or recommendation of the product to the exclusion of others that also may be suitable. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

Fig. 1. Enhanced efficacy of methionine (1 mM) and riboflavin (26.6 μ M) [M+R] mixture on powdery mildew control in 'Earliglow' strawberry by supplementation with surfactants. Pretreatments represent levels of disease severity before application of test compounds. Mean separation within each sampling was measured by Duncan's multiple range test; bars with the same letters were not significantly different at $P \leq 0.05$.

Fig. 2. Comparison of two surfactants in their effect on the efficacy of the methionine (1 mM) and riboflavin (26.6 μ M) [M+R] mixture in reducing powdery mildew in 'Earliglow' strawberry. Different letters indicate a significant difference within surfactant treatment as determined with Duncan's multiple range test at $P \leq 0.05$.

conidia suspension was sprayed onto the upper surfaces of the leaves to provide inoculum at ≈ 500 conidia/leaf. The plants were then placed in the greenhouse. Powdery mildew colonies became visible 7 to 10 d after inoculation. These plants were subsequently sprayed with a test solution until run off on a weekly schedule. The control plants were sprayed with distilled water. The first experiment was a study to determine the effect of various nonionic surfactants in combination with a mixture of 1 mM methionine and 26.6 μ M riboflavin (M+R) on efficacy of controlling powdery mildew in strawberry plants. Surfactants such as Tween 20, Triton X-100, sodium dodecyl sulfate (SDS) or Silwet L-77 (a nonionic silicone-polyether copolymer) at concentrations of 0.05% to 0.1%

were added in M+R mixture. The second experiment studied the effect of various metal ions on the efficacy of the M+R mixture. Supplements of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, ZnCl_2 , or $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, each at 1 mM, were made to the M+R mixture. The third experiment investigated the effect of free-radical scavengers (n-propyl gallate, thiourea) and antioxidants (α -tocophenol, β -carotene), at 0.1 mM each on the efficacy of the M+R mixture. The fourth experiment determined the effect of a mixture of methionine (1 mM), riboflavin (26.6 μ M), copper sulfate (1 mM) and the surfactants [sodium dodecyl sulfate (M+R+Cu+SDS mixture) or Silwet L-77 (M+R+Cu+Silwet L-77)] at concentrations ranging from 0% to 0.1% on controlling powdery mildew infections in strawberry. Throughout the studies, the plants used for each test solution were in a randomized complete block with six replications. Plants received weekly sprays for 3 weeks and the experiments were terminated after the fourth week. At the end of the experiments, the upper four expanded leaves were evaluated for the severity of powdery mildew infection. All experiments were duplicated.

The disease severity on the upper four expanded leaves were rated by the following classification: 0, no mildew lesions; 1, <5% leaf area colonized; 2, 5% to 20% leaf area colonized; 3, 20% to 50% leaf area colonized; and 4, >50% leaf area colonized. The disease severity was calculated according to the following equation (Tzeng et al., 1996b):

$$\text{Disease severity} = \frac{\sum(\text{disease rating} \times \text{number of leaves of that specific rating})}{4 \times 4} \times 100$$

where 4×4 represents the maximum rating of disease severity per test leaf (>50% leaf area colonized) $\times$ total of test leaves

Results and Discussion

Methionine and riboflavin are nontoxic essential nutrients for most living cells. The beneficial effects of foliar application of a mixture of methionine and riboflavin in reducing the severity of powdery mildew infection in strawberry plants were evident. Foliar application of a methionine and riboflavin mixture (M+R mixture) successfully controlled powdery mildew development on the leaves of the 'Earliglow' cultivar (Figs. 1–5).

The fungicidal activity of the M+R mixture on powdery mildew was enhanced by supplements of surfactants (Silwet L-77, SDS, Triton X-100, or Tween-20) (Figs. 1 and 2) and metal ions (Fig. 3). The presence of surfactants may alter energy relationships at interfaces, thereby reducing surface tension (Rosen, 1978) and enabling the applied agrochemical solutions to adhere to target surfaces more efficiently. Surfactants may also affect disintegrative membrane components and protein molecules (Tzeng et al., 1994) and enhance foliar absorption of biologically active compounds (Attwood and Florence, 1983). Thus, it is not unusual that supplementation of surfactants to the methionine and riboflavin mixture led to an enhanced fungicidal activity. SDS alone has been known to be toxic to certain microorganisms. It was generally believed that the toxicity was mainly due to the reduction of surface tension and a disintegrative effect on membrane components and protein molecules (Tzeng et al., 1994). Using powdery mildew of melon as a test model, Tzeng (unpublished data) recently demonstrated that surfactant (such as SDS, or Triton X-100) alone was effective to some extent in reducing disease severity. SDS at concentration of 1% also reduced powdery mildew infection in tobacco (Tzeng, 1988). In our study, the percentage of disease severity of strawberry plants 1 week after the third application with each individual surfactant were 40.7%, 45.3%, 66.0%, 39.5%, and 78.3% for SDS (0.1%), Triton X-100

Fig. 3. Effect of various metal ion (1 mM each) supplements in methionine (1 mM) and riboflavin (26.6 μ M) [M+R] mixture on controlling powdery mildew in 'Earliglow' strawberry plants. Pretreatments represent levels of disease severity before application of test compounds. Mean separation within each sampling was measured by Duncan's multiple range test; bars with the same letters were not significantly different at $P \leq 0.05$.

Fig. 4. Effect of the methionine (1 mM) and riboflavin (26.6 μ M) [M+R] mixture supplemented with free-radical scavengers or antioxidant compounds on biocidal activity for controlling powdery mildew in 'Earliglow' strawberry plants. Pretreatments represent levels of disease severity before application of test compounds. Mean separation within each sampling was measured by Duncan's multiple range test; bars with the same letters were not significantly different at $P \leq 0.05$.

(0.1%), Tween-20 (0.1%), Silwet L-77 (0.05%), and distilled water, respectively. The efficacy was, however, much less compared to the results obtained by using a combination of surfactants with the M+R mixture (Fig. 1). The disease severity of plants treated with M+R alone was $\approx 20\%$ to 30% . This variation may be due to environmental factors such as light intensity, light quality and chemical coverage. Tzeng and DeVay (1989) found that Gro-lux fluorescent light was superior to cool white fluorescent light and incandescent lights at the same light intensity in enhancing the biocidal activity of M+R. The possible reason for the effectiveness of Gro-lux fluorescent light compared to cool white fluorescent light or incandescent light was probably due to its richness of blue

light, the light region optimally absorbed by riboflavin (Tzeng and DeVay, 1989). The effect of M+R on experimental powdery mildews was a result of direct killing after contact with the chemical solution. If the applied chemical solution failed to cover certain plant parts, the fungicidal activity was not detected in those areas. Among the test surfactants, Tween-20 has long alkyl chains comprising its hydrophobic portion with a hydrophile-lipophile balance number (HLB) of 16.7. The HLB for Triton X-100 is 13.5. Triton X-100 has an alkylbenzene moiety as the hydrophobic portion and was more effective than Tween-20 as a supplement in M+R mixture for controlling powdery mildew infection (Fig. 1). Sodium dodecyl sulfate (SDS) not only has surfactant properties, but also can activate NADPH-dependent superoxide production in cell free systems (Bromberg and Pick, 1985), thus enhancing biocidal activity. The effectiveness of SDS as a surfactant in M+R mixture for controlling powdery mildew infection in strawberry was similar to

that of Triton X-100 (Fig. 1). Silwet L-77 was apparently the most effective in comparison to the other surfactants (Figs. 1 and 2). Weekly application of the M+R mixture, supplemented with 0.06% Silwet L-77, reduced disease severity to as low as 3% one week after the third spray. Silwet L-77 is an organosilicone-based surfactant with low surface tension (Loveland Industries, 1993). Silwet L-77 also has been reported to be less cytotoxic than a number of conventional nonionic carbon-based surfactants (Coupland et al., 1989), and to be rapidly hydrolyzed in acidic environments (Murphy et al., 1991). Therefore, Silwet L-77 could be used at a lower concentration than other surfactants in combination with the M+R mixture to control powdery mildew infection.

Supplements of CuCl₂ · 2H₂O, MgCl₂ · 6H₂O, FeCl₂ · 4H₂O, ZnCl₂, or MnCl₂ · 4H₂O, each at 1 mM, were added to the M+R mixture. These metal ions significantly increased the efficacy of the M+R mixture (Fig. 3). Cupric chloride and ferrous chloride were the two most effective metal ions in enhancing the fungicidal activity of the M+R mixture. Metal ions alone showed no significant effect on controlling powdery mildew infection (data not shown). However, with riboflavin as a photosensitizer and methionine as a reaction substrate, the mixture of M+R in the presence of light can generate various active oxygen radicals or active oxygen derivatives, including hydroxyl radical and singlet oxygen, which are strongly biocidal to a wide spectrum of microorganisms (Tzeng and DeVay, 1985, 1989, 1994; Tzeng et al., 1996a). In the M+R mixture, metal ions catalyze the Haber-Weiss reaction (Baker and Gebicki, 1986; Halliwell and Gutteridge, 1984, 1986) and result in continued generation of OH $\cdot$ (as shown below) which was previously shown to be a major factor contributing to the biocidal activity of the M+R mixture.

In the above reaction process, redox metal ions are oxidized and H₂O₂ is decomposed. The major role of O₂⁻ in the reaction appears to be the regeneration of H₂O₂ by autodismutation and the reduction of metal ions. For the reaction to continue, regeneration of H₂O₂ seems to be a rate-limiting step. Fe²⁺ or Cu²⁺ ion additive in the M+R mixture promoted H₂O₂ production. The disease control efficacy of the M+R mixture was not enhanced by increasing the

concentration of cupric chloride or ferrous chloride from 1 mM to 2 mM (data not shown). The inability to increase the efficacy may be because transition metal ions in the Haber-Weiss reaction are only catalytic. The addition of metal ions in the M+R mixture assured and accelerated the reaction process. The acceleration of the reaction process suggests a site-dependent rather than a concentration-dependent mechanism for these metal ions, a phenomenon typical for the toxigenicity of a metal ion-catalyzed oxygen radical generation system (Halliwell and Gutteridge, 1986). The fungicidal activity of the M+R mixture on powdery mildews depends greatly on the duration of the aqueous form of the applied M+R mixture. After drying-out of the aqueous form of the M+R mixture, the reaction for generating various active radicals diminished and the applied M+R are quickly absorbed by plant tissue and the photodynamic fungicidal activity dissipated. Therefore, the addition of metal ions is not as effective in enhancing the efficacy of the M+R mixture compared to the surfactants even though the metal ions may help to generate free radicals.

The presence of free-radical scavengers (n-propyl gallate, thiourea) and antioxidants (α -tocopherol, β -carotene), at 0.1 mM each reduced the toxicity of the M+R mixture for powdery mildew control (Fig. 4). The reduction of disease control efficacy by α -tocopherol may reflect the critical role of oxygen radicals in contributing to the biocidal activity. Antioxidative activity of α -tocopherol is due to its ability to trap the peroxy radicals, whereas that of β -carotene is due mainly to its ability to quench singlet oxygen (Burton and Ingold, 1984). Their effectiveness suggests that cell membranes are one of the primary targets of the biocidal activity.

Thiourea and n-propyl gallate are known to be efficient $\bullet$ OH radical scavengers. Supplement of thiourea or n-propyl gallate, each at 1 mM in concentration was effective in reducing the toxicity of the M+R mixture (Fig. 4). This provided further evidence for the direct involvement of free radicals in the photodynamic biocidal activity of the M+R mixture.

Fig. 5. Reduction of powdery mildew in 'Earliglow' strawberry plants by the mixture of methionine (1 mM) and riboflavin (26.6 μ M) [M+R] with or without supplementation of copper sulfate (1 mM) and surfactants (0.05 and 0.1%), SDS [M+R+Cu+SDS] or Silwet L-77 [M+R+Cu+Silwet L-77]. Pretreatments represent levels of disease severity prior to application of test compounds. Mean separation within each sampling was measured by Duncan's multiple range test; bars with the same letters were not significantly different at $P \leq 0.05$.

Methionine (1 mM), riboflavin (26.6 μ M), copper sulfate (1 mM), and surfactants (SDS or Silwet L-77 at concentrations of 0.05% to 0.1%) mixture [(M+R+Cu+SDS) or (M+R+Cu+Silwet L-77)] successfully controlled powdery mildew in strawberry plants (Fig. 5). Plants sprayed with a mixture of (M+R+Cu+SDS) or (M+R+Cu+Silwet L-77) exhibited none or only spotty infections at the end of the 3-week experimental period whereas the control (untreated) plants were severely colonized by the powdery mildew fungus. Silwet L-77 was more effective than SDS as a surfactant in the M+R+Cu mixture.

Results in this study suggest that the methionine and riboflavin mixture in combination with metal ions and surfactants may serve as an alternative to fungicides for controlling powdery mildew in 'Earliglow' strawberry. This treatment has also been applied to numerous seedling populations of strawberry with similar results (data not shown). The potential of using this MR mixture as a practical photodynamic pesticide appears to be promising, based on the experimental results.

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