Studies on Integration of Bactericide-resistant Biocontrol Agents into Conventional Copper-bactericide Sprays

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Abstract

To ascertain the feasibility of integrating a bactericide-resistant biocontrol agent into conventional bactericide sprays to control soft rot of cabbage under field conditions, detached petioles of Chinese cabbage were treated with either oxine-copper or basic copper sulfate followed by *Erwinia carotovora* subsp. *carotovora* strain 2TQ-19-9 or A-f-39. The survival rate of the more copper-resistant 2TQ-19-9 was higher after both treatments compared to the less resistant strain A-f-39. A rifampicin-resistant derivative of 2TQ-19-9, 2TQ-rif-4, was used for a subsequent field trial. Only 1.2% of the sensitive strains survived on Chinese cabbage plants treated in the field with oxine-copper as compared to 11.2% of the resistant strain one day after their application. Subsequent survival rates of the sensitive and resistant strains were not significantly different. A second application of the bactericide again reduced the population of the sensitive strain but not as much as did the initial application. This second application, however, caused an increase in the population of the resistant strain. The sensitive strain could not survive well after treatment, with the oxine-copper-bacterial mix spray and its population decreased to 2.25 cfu/g fresh weight of plant tissue. In contrast, the resistant strain survived to a final concentration of 1.06×10^3 cfu/g fresh weight of plant tissue.

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Key words : bactericide-resistant biocontrol agent, softrot disease, Chinese cabbage.

INTRODUCTION

Many investigators have reported about the efficacy of biological control in controlling plant diseases^{2,4,7,10}. One characteristic common to most bacterial biocontrol systems is inconsistency in disease control, which hampers the commercialization of most systems. Weller reported that in commercial-scale tests, pseudomonads that suppress take-all and *Pythium* root rot significantly improved yield only 60% of the time⁹. Weller *et al.*⁹ suggested that applying a combination of strains might improve the consistency of the field performance of biocontrol systems.

By extrapolation, copper-resistant biocontrol agents could perhaps be integrated into conventional copper sprays to improve their consistency^{2,3} and to reduce the use of copper sprays. In our previous paper, we reported the isolation of bactericide-resistant biocontrol agents which survived in solutions of this bactericide⁶. We hereby report the survival of this agent in integrated

bactericide sprays not only on detached petioles of Chinese cabbage, but also under field conditions. Preliminary results of this work have already been reported⁹.

MATERIALS AND METHODS

Bacterial cultures All the bacterial strains used in this work are avirulent derivatives of *Erwinia carotovora* subsp. *carotovora* pathogenic strain 2T-2. Bactericide-resistant strains 2TNQ-19-9 and A-f-39 were used for the test on detached petioles of Chinese cabbage. 2TNQ-19-9 is resistant to 4 g/l and 2 g/l (250- and 500-times dilution) of oxine-copper (80% WP) and basic copper sulfate (42% WP), respectively⁶, whereas A-f-39 is resistant to 1 g/l (1000-times dilution) of oxine-copper on PDA plate but sensitive to the basic copper sulfate. A rifampicin-resistant derivative of 2TNQ-19-9, designated as 2TQ-rif-4, with similar characteristics as the parent was used for the field trial. Also, a rifampicin-resistant mutant of strain 2T-2 designated as 2T-rif-1 (virulent) was used as a control.

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Bacterial strains were grown overnight at 28°C on nutrient agar slants (meat extract, 10 g; polypeptone, 10 g; NaCl, 5 g; agar, 13 g and distilled water, 1 liter; pH 7) and transferred into nutrient broth (same components as nutrient agar but without agar), incubated with shaking overnight at 28°C. Bacterial cells were then washed by centrifugation and suspended in an appropriate volume of sterile distilled water to a concentration of 10^7 – 10^8 cfu/ml.

Chemicals used Wettable powder of oxine-copper (Quinondo; Cu-8-hydroxy-quinolin, 80%) and basic copper sulfate (Z-Bordeaux; Cu, 42%) were used for the test on detached leaves. In the field trial, only the oxine-copper was applied.

Bactericide resistance on detached petioles Fresh, healthy petioles of Chinese cabbage were trimmed to 7×15 cm. The petioles were not washed in order to maintain a micro-environment similar to those under field conditions. The adaxial side of the petioles were sprayed with 2 g/l (500-times dilution) of either basic copper sulfate or oxine-copper. The control samples were sprayed with sterile distilled water. The sprayed petioles were air-dried and later sprayed with the respective bacterial suspension (10^7 – 10^8 cfu/ml) of either A-f-39 or 2TQ-19-9. The petioles were incubated at 25°C in a moisture chamber. At each sampling time, five disks (1.9 cm diameter) were taken from a detached petiole for each treatment. The disks were cut using a sterile cork-borer, and the abaxial portion was removed with a sterile spatula and shaken mechanically in 100 ml sterile distilled water for 10 min. This treatment aims at avoiding contaminants present on the abaxial side of the petioles. One ml of the resulting solution was serially diluted, plated on modified Drigalski's medium (meat extract, 10 g; polypeptone, 10 g; lactose, 10 g; crystal violet (0.1%), 5 ml; bromothymolblue (BTB, 0.2%), 40 ml; agar, 13 g and distilled water, 1 liter; pH 7) and incubated at 28°C for at least 3 days. The resulting yellowish colonies were then counted. The samples taken after 2 hr incubation were designated as samples taken after day 0 of incubation. Later, samples were taken after 1, 2, 4, 6, and 7 days of incubation.

Field trials Chinese cabbage plants, sown on 24 May, were transplanted to the field 2 weeks later at 45-cm spacing in drills 75 cm apart resulting in 28–30 plants per row. Eight rows were used for this experiment: three rows for each bacterial strain with a border row on each side. Plants were sprayed with either bacteria alone, one application of oxine-copper with a bacterial spray on the next day, or an oxine-copper-bacterial mixture. The oxine-copper-bacterial solution was applied immediately after preparation. The concentration of bacterial cells used were 3.6×10^7 and 1.4×10^8 cfu/ml for the resistant and sensitive strain, respectively. Half the rows in the staggered spray of oxine-copper and the bacterial treatment was subjected to a second application of oxine-copper, one week after the 1st spray. With the exception of this, all other treat-

ments were applied once. The oxine-copper was used at 0.83 g/l (1200-times dilution) according to the manufacturer's recommendation. During the spraying, a vinyl sheet was held between the rows to avoid contaminating other rows. The oxine-copper was applied on 8 June. Bacterization was done on 9 June, but only the bacterial suspension treatments were sampled on that day (day 0). Subsequent samplings were done on days 1, 2, 7, 14 and 21 after bacterization. Three plants from each treatment were harvested for each sampling, and 5–7 cm of the stem section were excised (avoiding the roots and soil particles) and bulked to represent each treatment. The bulked sample (300–400 g fresh weight) for each treatment was mechanically shaken in 300 ml sterile distilled water for 10 min, and 1 ml of the resulting solution was serially diluted and plated on modified Drigalski's medium containing 100 µg/ml rifampicin. The plates were then incubated at 28°C for 3 days, and the yellowish colonies that appeared were counted.

RESULTS

Bactericide resistance on detached petioles

The population of the resistant strain A-f-39 at day 0 of incubation was 5.5×10^5 cfu/cm² on the control petioles and gradually decreased to 1.7×10^4 cfu/cm² petiole after 7 days of incubation (Fig. 1). Petiole samples taken at day 0 from the oxine-copper and basic copper sulfate treatments had populations of 1.1×10^4 and 1×10^5 cfu/cm² petiole, respectively. These numbers represent survival rates of 2.0% and 18.2% compared to the bacterial population of the control plot. A-f-39 was not detected during subsequent sampling on the petioles treated with basic copper sulfate. The population of

Fig. 1. Survival of strain A-f-39 on petioles of Chinese cabbage treated with 2 g/l (500-times dilution) of oxine-copper or basic copper sulfate.

Fig. 2. Survival of strain 2TQ-19-9 (resistant mutant) on petioles of Chinese cabbage treated with 2 g/l (500-times dilution) of oxine-copper or basic copper sulfate.

A-f-39 in the oxine-copper treatment was also below detection (2.0 cfu/cm^2 petiole) after days 1 and 2 of incubation but subsequently, the population grew to 10^3 – 10^4 cfu/cm^2 petiole after days 5 and 7 of incubation.

The population of 2TNQ-19-9 on the control petioles at day 0 was $4 \times 10^5 \text{ cfu/cm}^2$ petiole (Fig. 2). Samples taken from petioles treated with oxine-copper and basic copper sulfate at the same sampling time had populations of 1.1×10^5 and $1.7 \times 10^5 \text{ cfu/cm}^2$ petiole, respectively. These represent survival rates of 27.5% on petioles treated with oxine-copper and 31.4% on basic copper sulfate-treated petioles. The subsequent populations of 2TNQ-19-9 on petioles treated with either oxine-copper or basic copper sulfate fluctuated significantly over time, though they were never below the detection level. In a preliminary work using detached leaves from younger Chinese cabbage plants treated with oxine-copper and the bacteria, strain 2TNQ-19-9 could also survive oxine-copper sprays under field conditions. This led us to repeat this experiment under normal cultural practices.

Field trials

The population of the sensitive strain 2T-rif-1, persistently decreased, from 1.4×10^6 to $3 \times 10^2 \text{ cfu/g}$ fresh weight of plant tissue in the control treatment within 21 days of sampling (Fig. 3). On samples from the staggered sprays of oxine-copper and bacterial suspension, only 1.5% of the sensitive strain survived after the 1st day of bacterization as compared to the samples in the control treatment. Subsequent samples were not significantly different in this treatment, except that the population of the sensitive strain 2T-rif-1, increased 7 days after the bacterization, akin to that in the control treatment. The second application of oxine-copper reduced the popula-

Fig. 3. Survival of strain 2T-rif-1 (sensitive) in the various combinations of treatments with oxine-copper on field grown Chinese cabbage plants (Arrows indicate days on which the oxine-copper was applied).

Fig. 4. Survival of strain 2TQ-rif-4 (resistant) in the various combinations of treatments with oxine-copper on field grown Chinese cabbage plants (Arrows indicate days on which the organic copper was applied).

tion of the sensitive strain by about 10 times, but total elimination was not possible; the population 14 days after the treatment was even higher than the control.

The oxine-copper-bacterial solution treatment decreased the population of the sensitive strain from 1×10^5 (cell numbers of control were estimated at the same time) to $4.6 \times 10^2 \text{ cfu/g}$ fresh weight of plant tissue 1 day after bacterization. Subsequently, the cell numbers de-

creased further to 2.9 cfu/g fresh weight of plant tissue 21 days after this treatment.

The initial concentration of the bactericide-resistant strain 2TQ-rif-4 in the control treatment was $3.6 \times 10^5 \text{ cfu/g}$ fresh weight of plant tissue, which decreased to $3 \times 10^3 \text{ cfu/g}$ fresh weight of plant tissue 21 days after bacterization (Fig. 4). Samples from the staggered spray of oxine-copper and the bacterial suspension after the 1st day of bacterization showed that 11.6% of the resistant strain survived in comparison to the control. The second application of oxine-copper seemed to benefit the population of strain 2TQ-rif-4, leading to an increase in its population 7 days after this treatment.

Although the population of the resistant strain in the oxine-copper-bacterial treatment was slightly below that of the staggered treatment of the bactericide and bacterium on days 2 and 14 after bacterization, this slight decrease was overshadowed by an increase in cell numbers after day 21. A similar experiment to assess the correlation of the population of the bactericide-resistant strain with populations of other foliar microbes also showed that the mutant 2TQ-rif-4 increased to 2.9×10^4 after the second application of oxine-copper as compared to 2.8×10^3 for the single oxine-copper application treatment at the same sampling time. This was two times higher than that found in the control ($1.02 \times 10^4 \text{ cfu/g}$ fresh weight of plant tissues) where only the bacterium was applied. Subsequent sampling also showed the same pattern as observed in Fig. 4.

DISCUSSION

The difference in populations between the oxine-copper-resistant mutant and the sensitive strain after day 0 of incubation could be attributed mainly to the differences in their resistance against the toxic action of this bactericide. The inability to detect A-f-39 after days 1 and 2 of incubation can be explained by its lower resistance to the oxine-copper (1.0 g/l) as well as by its sensitivity to basic copper sulfate compounded by the sensitivity of the dilution plate method (2.0 cfu/cm^2). Its apparent growth surge after days 5 and 7 of incubation may be due to a decrease in the active components of the bactericide to a level that can be tolerated by this strain or to an increase in the strain's resistance. The higher survival rate of A-f-39 after day 0 of incubation in the basic copper sulfate treatment is explained by the fact that the duration or contact was too short for the bactericide to act effectively on the bacterial cells. This point was supported by the results of subsequent samplings, as no bacterial cells were detected.

Conversely, strain 2TQ-19-9 exhibited a relatively higher survival rate on petioles treated with either the oxine-copper or the basic copper sulfate throughout the period of sampling. This higher survival rate confirms the resistance of this mutant to these bactericides on PDA plates and indicates that 2TQ-19-9 could be integrated into field sprays of these bactericides. The

fluctuation in cell numbers of 2TQ-19-9 on the petioles treated with the bactericides could be attributed to the effect of these bactericides on the growth of this bacterium because no such fluctuation was observed in the control treatment.

The results described led us to try rifampicin-resistant derivatives of strain 2TQ-19-9 to check the feasibility of incorporating it into oxine-copper sprays under field conditions. The 11.6% survival rate of 2TQ-rif-4 on field grown Chinese cabbage plants treated with the oxine-copper after day 1 of bacterization is comparable to the results (5–10% survival) obtained by Anderson *et al.* for resistant strains of *P. syringae* on plants treated with copper hydroxide (Kocide 101 at a rate of 1.5 g/l)¹⁾. The lower survival rate of the sensitive strain 2T-rif-1 (1.2%) during the same period, despite its initial high inoculum concentration, affirms its sensitivity to oxine-copper.

The second application of the bactericide was not effective in reducing the number of 2T-rif-1 cells to the same extent as the initial application, in conformity with the observation of Anderson and his co-workers¹⁾ that the bactericide, copper hydroxide (1.2 g/l), was less effective at killing established populations of *P. syringae* irrespective of their degree of resistance. Applied copper-based bactericides may therefore be generally ineffective against established bacterial strains, including phytopathogenic ones. Sufficient monitoring of the pathogens and critical timing of bactericide applications may therefore be crucial in ensuring high efficacy of bactericides in disease control programs. Also underscored is the importance of integrating bactericide-resistant biocontrol agents into bactericide sprays to ensure effective disease control. The second application of the bactericide seems to increase the population of the resistant strain, which may improve their efficacy as biological control agents.

The oxine-copper-bacterial mixture was used to examine the effect of such a direct integration of the bacteria with the chemicals on the applied bacterial strains. The resistant strain applied in this way was able to survive the bactericide and attained cell populations comparable to that in the staggered treatment, whereas the sensitive strain exhibited both poorer survival and growth. Preliminary results indicated a reduction in the disease severity by the combined sprays of the biocontrol agent and the oxine-copper compared to the application of the agent as a single treatment, but a more detailed work is needed for further confirmation.

We can, however, conclude that strain 2TQ-rif-4 could be integrated into oxine-copper sprays in the field either as a mixture or in staggered applications. To integrate sensitive biocontrol agents into copper-based bactericide sprays, such agents could also be applied first to get established before applying the bactericide. Such integrations may be a key to a safer environment without compromising the effectiveness of disease control programs.

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和 文 摘 要

Ampaabeng Gyedu KYEREMEH・菊本敏雄・荘 敦堯・高原吉幸・江原淑夫: 銅剤耐性生物防除用菌株と銅剤との併用の可能性に関する研究

銅剤耐性の生物防除用菌株を作出し、この耐性菌と銅剤との併用による軟腐病防除の可能性について実験を行った。はじめに、ハクサイの切離葉に500倍の銅水和剤と有機銅水和剤をそれぞれ散布し、風乾後に銅剤耐性および感受性の非病原性軟腐病菌(10^8 cfu/ml)を散布し、25°C湿室中に静置して経時的に菌数の変動を調べた。耐性菌の方が高い生残率を示した。前日に所定濃度(1200倍)の有機銅水和剤を散布しておいた圃場のハクサイに、両菌より分離したリファンピシン耐性菌を散布した。散布1日後の生残率は銅剤耐性菌の方が感受性菌より10倍高かった。しかし、その後の両菌の菌数はほぼ同様の推移を示した。これに対し、有機銅水和剤に直接菌を懸濁して散布した場合、感受性菌の生残率は著しく低くなった。つぎに、1週間後に再度銅剤を散布し、その影響を調べた。その結果、ハクサイに定着した耐性菌はまったく銅剤散布の影響を受けず、銅剤との併用が可能であることが明らかになった。