Application of a bio-control agent for controlling strawberry anthracnose in Taiwan

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ABSTRACT

Strawberry (*Fragaria* × *ananassa* Duchesne) is an economically important crop in Taiwan, however because of the outbreak of strawberry anthracnose in early 2010, the short supply of strawberry seedlings became a serious problem for strawberry production. The isolates of anthracnose causing organisms were collected from major strawberry cultivation areas in Miaoli County, and mainly identified as *Colletotrichum siamense*, these isolates were proved to be pathogenic to the leaves, crown and roots of strawberry. The bio-control agent of *Bacillus amyloliquefaciens* P-2-2, was isolated from rice paddy field and expressed effectively on inhibition of many fungal pathogens of strawberry. The strawberry seedlings used in this study were treated with 400-fold dilution of *B. amyloliquefaciens* P-2-2 fermentation broth once for every two weeks to protect the crown of the seedlings from anthracnose infection before transplanting. Sterilization of soil by biological soil disinfestation method was done during field preparation. After transplanted in the production field, the strawberry transplants were continuously treated with 400-fold dilution of *B. amyloliquefaciens* P-2-2 once for every two weeks until the end of the growing season. The results of field test, shown that the survival rate of the untreated group in demonstration area of Dahu Township was 83.9%, whereas the survival rate of seedlings treated with *B. amyloliquefaciens* P-2-2 increased to 95.1%. The replanting rate of strawberry in the untreated group area was 37.6%, compared to the *B. amyloliquefaciens* P-2-2 treated area which was reduced

significantly to 10.7%. Upon those results indicated that application of bio-control agents with other practices were effective for controlling strawberry anthracnose.

Keywords: Taiwan, strawberry, anthracnose, bio-control agent, biological soil disinfestation

INTRODUCTION

Strawberry is an important economic crop in Taiwan. Although the total domestic production area is only about 550 hectares, of which 90% is located in Miaoli County, the total value of fresh and processed fruits exceeds 1.8 billion of New Taiwan Dollar (NTD). If the strawberry seedlings needed for transplanting at 45,000 plants per hectare, the total annual demand will reach 25 million plants. However, in recent years, due to the occurrence of strawberry anthracnose, the supply of strawberry seedlings is insufficient. In addition to the abnormal climate, the temperature is still very high (above 25^{o}C) right after transplanting of strawberry seedlings in September through October and November, resulting in transplanted strawberry seedlings damaged by anthracnose with gangrenous crown and withering and dying leaves. The replanting rate is as high as 30 to 40%, which causes a big crisis in the strawberry industry. It is urgent to solve the problem of strawberry anthracnose.

This study intends to use the biocontrol agent and soil disinfection methods to prevent the occurrence of anthracnose disease in strawberry seedling stage and field cultivation period. Because strawberries suffer from many pests and diseases during the seedling stage and field cultivation period, farmers often applied chemical pesticides to control these problems. Countries such as the United States, Japan, etc., cannot completely achieve the economic production of strawberries without application of chemical pesticides. Through the biocontrol agent and health management during this study, we might have the opportunity to lead the world in the production of organic, pesticide-free strawberries.

The techniques for integrated pest management of strawberry

The techniques for integrated pest management of strawberry during vegetative and flowering stage developed by TARI describe as following.

1. Pre-treatment of strawberry seedlings before transplanting: Before transplanting, strawberry seedlings were soaked at 1000-fold dilutions of "Anthracnose no trace", "Black Asura" and 200-fold dilution of vegetable oil mixture to reduce the contaminated primary inoculum of pathogens and insects into the field.
2. Pest management in strawberry vegetative and fruiting stages:

a. Disease management: The main diseases of strawberry are fruit rot caused by *Phytophthora* spp., powdery mildew disease by *Sphaerotheca aphanis* and gray mold disease by *Botrytis cinerea*. Before the rainy season, the naturalized phosphorous acid (1000-fold dilution) is applied once a week for three times to prevent the occurrence of fruit rot. Emulsified sunflower oil (200 to 500-fold dilution) is applied at the beginning of powdery mildew and gray mold. The "vigor energy" (1000-fold dilution) is applied for controlling anthracnose disease.
b. Pest management: Pests include *Tetranychus urticae*, *Aphis ichigocola*, *Spodoptera litura*, *Scirtothrips dorsalis*, and *Frankliniella intonsa*. Among them, *Frankliniella intonsa* mainly causes harm at flower organs during flowering stage. The other four pest species may occur year-round. It is necessary to follow the actual monitoring data of pests and diseases in order to master the appropriate methods and timing of control measures. Samples of the old and middle leaves, and young shoots were monitored. At the nursery and before flowering in the field, strawberries were sprayed with the vegetable oil mixture to inhibit the populations of spider mites. At the beginning of flowering stage, the biological natural enemies "*Mallada basalis*" were released to control the two-spotted spider mites and strawberry aphid, and the "*Orius* spp." was released to control the thrips (*Scirtothrips dorsalis* and *Frankliniella intonsa*). Each plant received about 10 eggs or 5 larvae of the first instar of "*Mallada basalis*", once every 7 to 10 days until the late harvest. The invasion of *Spodoptera litura* can be controlled by spraying *Bacillus thuringiensis* if needed. If the insect pest is found to be serious and the control efficacy of natural enemies and vegetable oil mixture are not sufficient, then the recommended chemical pesticides in the plant protection manual will be used for prevention and control.

3. Field sanitation: Weeds in and around the strawberry filed must be removed. The debris of unwanted branches, leaves, flowers, and fruits should not be left in the field, for they may serve as the habitat for the growth and proliferation of pathogens and pests. The unwanted strawberry leaves should be removed and placed in the bag and taken away from the field. They should be immediately burned or buried under the ground for at least 30cm deep, and should not be disposed in the field or in the drainage ditch.

Identification of strawberry anthracnose

Strawberry anthracnose is an important disease in the cultivation of strawberry. The pathogen could infect the leaves, fruits, petioles and stolon and cause dark brown sunken lesions on strawberry. The pathogen can also damage the crown of strawberry, causing symptoms like browning of the vascular bundle and withering of the strawberry

plants. Strawberry anthracnose pathogen often infects strawberry at the seedling stage and causes signs of withering between the late seedling stage and the early stage after transplanting. It can even cause the death of strawberry seedlings, resulting in the lack of seedlings as transplanting supplies. The anthracnose not only increases the cost of strawberry cultivation, but also affects the quality of the fruit, so the detection of pathogens and the establishment of a healthy seedling system are crucial [8]. The purpose of this study was to identify the pathogens of strawberry anthracnose to facilitate and monitor both in nursery and in filed diseases for better management. The anthracnose isolates were collected from major strawberry cultivation areas such as Dahu Township, Miaoli County and Guoxing Township, Nantou County. A total of 20 isolates collected from 2010 to 2018 were identified by using specific primers designed for *Colletotrichum acutatum*, *C. gloeosporioides* and *C. boninense* species complex [7, 9, 12, 14]. The *C. gloeosporioides* species complex was further identified by using specific markers [3] that could discriminate between *C. fructicola*, *C. aenigma*, and *C. siamense* within *C. gloeosporioides* species complex. The results showed that 18 isolates were *C. siamense*, one isolate was *C. fructicola*, and another one belonged to *C. gloeosporioides* species complex, but specific species could not be identified. According to the primary result, *C. siamense* was the main species that caused strawberry anthracnose in Dahu Township and Guoxing Township (Fig. 1).

The pathogenicity test of C. fructicola and C. siamense

C. fructicola and *C. siamense* were originally isolated from the diseased crown and leaf of strawberry, respectively. The cultures of *C. fructicola* and *C. siamense* were grown on PSYA medium for 5-7 d, the cultures were then flooded with distilled water to wash off the spores; this was followed by filtration through a single layer of Miracloth (Calbiochem, San Diego, USA) to remove any mycelial residue from the conidial suspension. The final concentration of the suspension was adjusted to 1 x 10^1, 10^3 and 10^5 spores/mL and was used for the experiments of both inoculation and spore germination. The leaves, crown and roots of strawberry were inoculated with different inoculation methods. The roots were soaked in spore suspension after the root tips were freshly cut; the damaged crowns were drench-inoculated with spore suspension after petioles were removed. The leaves were spray-inoculated with spore suspension. The inoculated strawberry plants were kept in a moisture chamber for one day before they were moved to greenhouse for one week for symptom observation. The results showed that *C. fructicola* was pathogenic to the crown and roots but not to the leaves of strawberry plants (Fig. 2), whereas *C. siamense* was pathogenic to the leaves, crown and roots (Fig. 2). The primary results showing different pathogenicity between two

anthracnose species might indicate that strawberry anthracnose disease in Taiwan was mainly caused by *C. siamense*.

Antagonistic test of biocontrol agent against fungal pathogens

Previous studies indicated that biocontrol agent, *Bacillus amyloliquefaciens* P-2-2, was able to induce disease resistance in vegetable crops. The pathogens (*Colletotrichum siamense*, *Rhizopus* sp., *Sclerotinia sclerotiorum*, *Pythium* sp., *Fusarium* sp. and *Botrytis cinerea*) were isolated from diseased strawberry plants. The inhibitory effect of *B. amyloliquefaciens* P-2-2 on mycelial growth of fungal pathogens of strawberry was conducted by dual culture of the respective pathogens and *B. amyloliquefaciens* P-2-2 on 1/2PDA+1/2NA medium, and the mycelial length were recorded and the inhibition rate was calculated. The inhibition rate of *B. amyloliquefaciens* P-2-2 against *C. siamense*, *Rhizopus* sp., *S. sclerotiorum*, *F. oxysporum* and *B. cinerea* was 45.76, 16.91, 44.75, 8.45, and 42.05%, respectively. The results indicated that application of *B. amyloliquefaciens* P-2-2 to strawberry plants could not only effectively inhibit the anthracnose pathogen and but also to other diseases caused by other tested fungal pathogens (Fig. 3).

Control efficacy of biocontrol agent against strawberry anthracnose in greenhouse

The healthy strawberry seedlings (varieties Toyonoka and Tianlai No.1) provided by the farmers of Dahu, Miaoli county. The beneficial microorganism *B. amyloliquefaciens* P-2-2 was used to test its control efficacy on strawberry anthracnose. Once the strawberry seedlings were established in the greenhouse, their leaves were sprayed with different dilution of *B. amyloliquefaciens* P-2-2 fermentation broth one day before they were inoculated with anthracnose pathogen. A total of four groups, distilled water and 200-, 400-, and 600-fold diluted *B. amyloliquefaciens* P-2-2 fermentation broth were used to test their inhibitory efficacy on the control of anthracnose disease. After being inoculated with spores ($1x10^5$ spores/mL) of anthracnose pathogen, the strawberry seedlings were maintained in the acrylic box for one day before the cover was removed. The strawberry seedlings were then transferred to greenhouse for 7-14 days. The numbers of the anthracnose lesions were recorded. It was found that 200-fold and 400-fold diluted *B. amyloliquefaciens* P-2-2 fermentation broth were able to inhibit the formation of anthracnose lesions on Toyonaka variety. It was found that 400-fold and 600-fold diluted *B. amyloliquefaciens* P-2-2 fermentation broth were able to inhibit the formation of anthracnose lesions on Tianlai No.1 variety.

The best dilution rate of *B. amyloliquefaciens* P-2-2 fermentation broth was 400-fold for both tested strawberry varieties (Fig. 4).

The methods for disinfection of irrigation water

Many methods are available for disinfection of irrigation water including filtration, heat treatment, oxidizing agents, and ultraviolet radiation. Plant pathogens showed different sensitivity to different disinfection methods [1, 4, 6, 15, 16]. Chlorine, as either calcium hypochlorite or sodium hypochlorite, is commonly used to treat irrigation water because it is inexpensive and easy to apply. When chlorine is introduced to water, it reacts to form free chlorine species of hypochlorous acid (HClO) or hypochlorite (ClO^-) ions, depending on the pH of the water, which oxidize organic materials, including any pathogens present in the water [17]. Hypochlorous acid is the stronger, faster oxidizer, but is more prevalent when the pH of the water is between 6.5 and 7. As the pH of water increases, the hypochlorous acid is converted to hypochlorite, which is a weaker oxidizer and disinfectant. The disinfection efficacy of hypochlorous acid is 80 times higher than that of sodium hypochlorite. The inhibition effect of hypochlorous acid on germination of anthracnose spores was conducted. Aliquots (5 μL) of hypochlorous acid solutions were dropped onto glass slides and mixed with an equal volume of conidial suspensions (2×10^4 spores/mL), to make final concentrations of hypochlorous acid of 0.05, 0.25, 2.5, 5, 25 and 50 ppm, with a final concentration of the conidial suspension set at 1×10^5 spores/mL. DW was used as a control. The germination of conidia was observed and recorded 16 h after incubation. The inhibition rates of 50, 25, 5, 2.5, 0.5, 0.25, and 0.05 ppm hypochlorous acid solution on the spore germination of anthracnose pathogen were 100, 95.07, 85.20, 65.70, 52.47, 61.77, and 59.20%, respectively (Fig. 5).

The methods for disinfection of cultural medium and field soil

Methyl bromide had been rated as the most effective chemical fumigant for many plant diseases and pests [2, 5]. However, it destructively damages the ozone layer which in turn makes the sunlight become more harmful to human health [2]. It has been prohibited for agricultural use under the law; therefore, some soil-borne diseases have become more difficult to control by other available measures. Other soil sterilization methods, such as soil solarization and flooding, required high temperature and a long incubation period [2, 5]. A new method, named as biological soil disinfestation (BSD), developed by Shinmura [10, 11], fortified by soil reduction, which required neither high temperature nor a long incubation period, consisted of following three steps: (1) introduction of easily decomposable organic materials e.g., molasses, rice bran, rice

straw, wheat bran, etc., at a rate of 100-200kg/acre of soil, (2) flooding the soil by irrigation, and (3) covering the soil surface with plastic film to maintain the reduced soil conditions. BSD using molasses or wheat bran proved to be effective against a wide range of soilborne pathogens, such as *Fusarium oxysporum*, *Ralstonia solanacearum*, and *Verticillium dahliae*, as well as the nematodes *Meloidogyne incognita* and *Pratylenchus* sp. [10, 11, 13].

Disinfection of strawberry cultivation medium (such as peat moss and coconut fiber) was accomplished by the steam produced by water continuously heated by three cascaded gas water heaters (Fig. 6). Sterilization of soil by biological soil disinfestation (BSD) (Fig.7) with the introduction of easily decomposable organic materials to soil, flooding the soil by irrigation, and covering the soil surface with plastic film to maintain the reduced soil conditions. Application of *Bacillus amyloliquefaciens* P-2-2 to the cultivation medium and fields after disinfection (Fig. 8) to occupy the niche of pathogens of anthracnose and Fusarium wilt of strawberry was conducted.

Using beneficial microorganism and hypochlorous acid to cultivate health strawberry seedlings

In order to cultivate healthy strawberry seedlings, the tissue-cultured strawberry seedlings that are free of any specific pathogens were provided to the farmers to be used as mother plants. They were treated with the application of 400-fold diluted *B. amyloliquefaciens* P-2-2 fermentation broth to protect the crown of strawberry seedlings from the infection of anthracnose spores. Lastly, they were sprayed with the hypochlorous acid solution daily to kill the anthracnose spores disseminated from the diseased tissues of the seedlings (Fig. 9).

The result of field test

The survival rate of the untreated group in demonstration area of Dahu Township was 83.9%, whereas that of healthy strawberry seedlings treated with *B. amyloliquefaciens* P-2-2 broth increased to 95.1%. The replanting rate of strawberry in the untreated group area was 37.6% (Fig. 10), whereas that of the *B. amyloliquefaciens* P-2-2 treated area was reduced to 10.7% (Fig. 11). The use of *B. amyloliquefaciens* P-2-2 for 6 months increased the cost by no more than NTD 1,800, but it reduced the loss of about NTD 25,300 (including about NTD 20,500 for replanting strawberry seedlings and NTD 4,800 for labor wages). In addition, the use of biocontrol agent to grow healthy strawberry seedlings allows the growers to transplant the seedlings into the field in mid-September (Fig. 12). Other farmers, who do not use these technologies, fear that due to the shortage of strawberry seedlings, or high temperature, could only transplant

the strawberry seedlings until the end of October, which would delay strawberry harvest time and lose the good marketing opportunities.

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18.

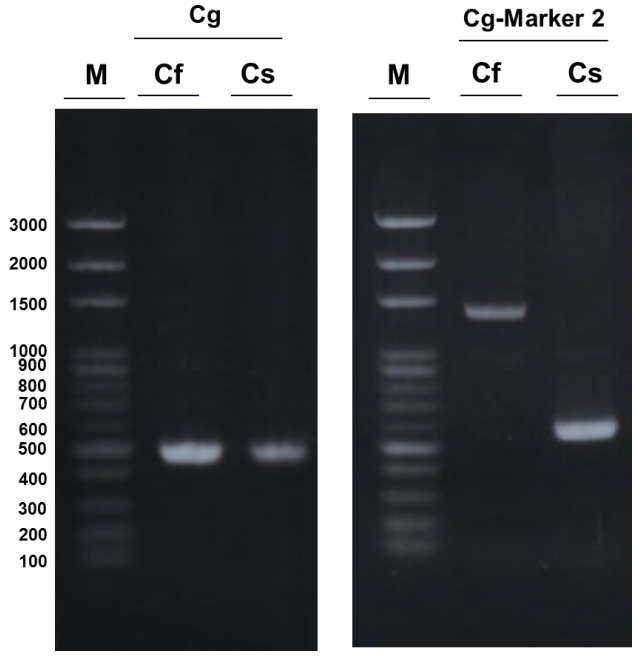


Fig. 1. Identification of strawberry anthracnose with primer for *Colletotrichum gloeosporioides* species complex and specific markers.

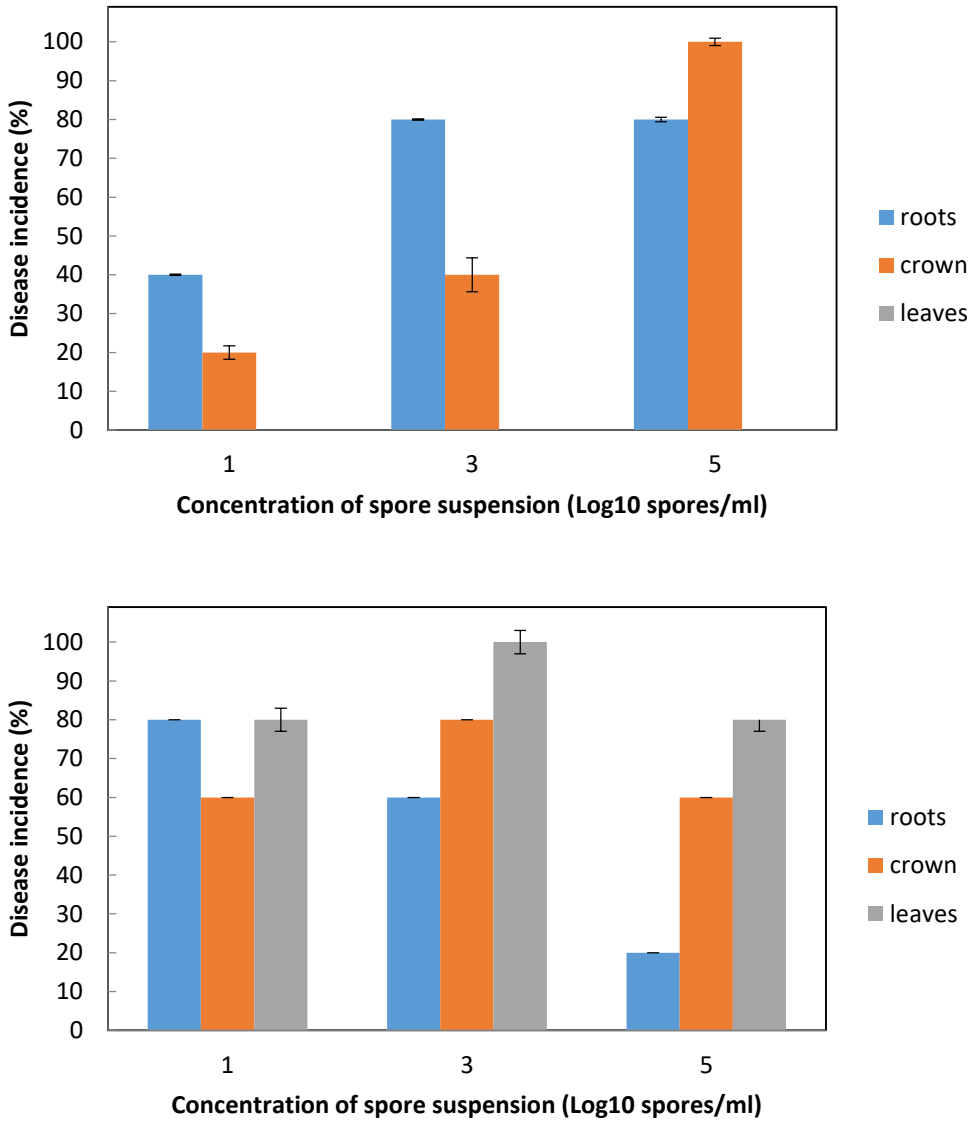


Fig. 2. Pathogenicity test of *C. fructicola* (upper) and *C. siamense* (lower).

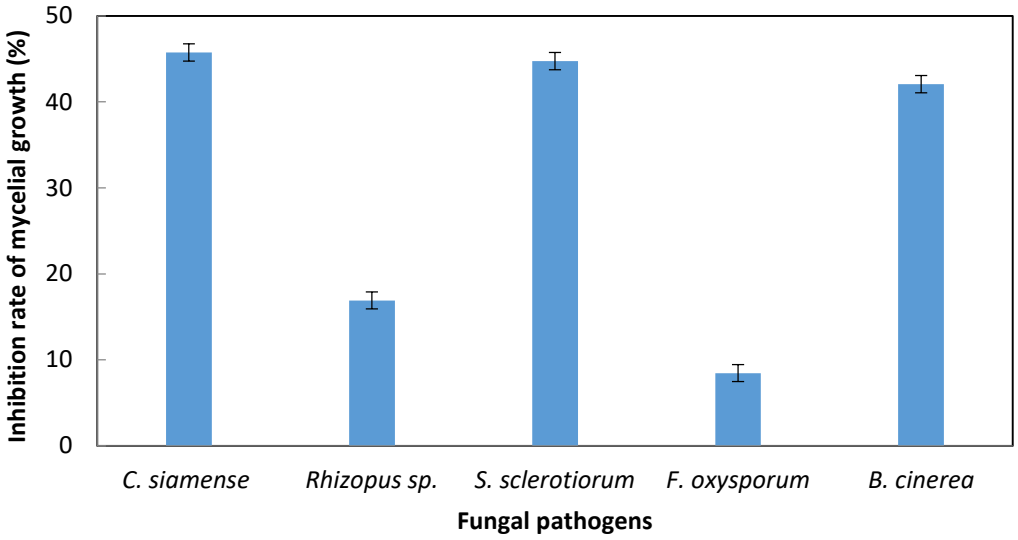


Fig. 3. Antagonistic test of biocontrol agent against fungal pathogens.



Fig. 4. Control efficacy of biocontrol agent against anthracnose on two strawberry varieties in greenhouse.

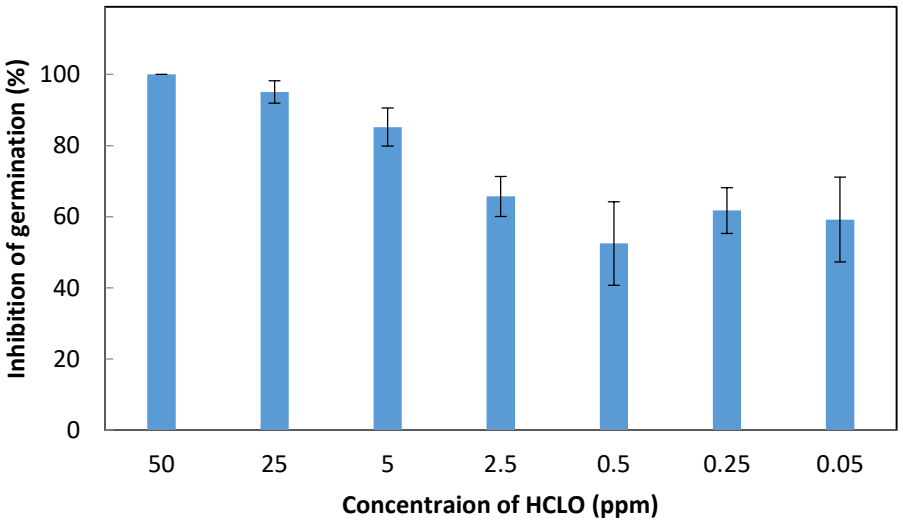


Fig. 5. The inhibitory effect of hypochlorous acid (HClO) on the spore germination of strawberry anthracnose pathogen.



Fig. 6. The gas water heaters used to generate steam for the disinfection of cultivation medium for strawberry.



Fig. 7. Soil covered with plastic film for disinfection; soil was added with organic matter followed by being flooded with water before being covered with plastic film.



Fig. 8. The biocontrol agent was applied to the disinfected soil.



Fig. 9. Propagation of healthy strawberry seedlings treated with biocontrol agent and hypochlorous acid.



Fig. 10. Many dead strawberry seedlings due to anthracnose disease in the untreated field.



Fig. 11. Healthy strawberry seedlings effectively protected from anthracnose disease with the treatment of biocontrol agent and soil disinfection.



Fig. 12. Strawberry seedlings cultivated and protected with the biocontrol agent against the anthracnose disease and were transplanted in mid-September.