Biomatriconditioning or biopriming with biofungicides or biological agents applied on hot pepper (*Capsicum annuum* L.) seeds reduced seedborne *Colletotrichum capsici* and increased seed quality and yield

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Abstract

Matriconditioning, controlled seed hydration by moistened carriers with high water adsorptive matric forces, has been proven to improve seed viability and vigor, plant growth and yield of various crops. Bioprotectants and/or chemical pesticides can be used in combination with matriconditioning, the process being biomatriconditioning. Biomatriconditioning with the biofungicide clove oil 0.06% or 0.1% were effective seed treatments to improve the vigor and relative speed of germination while reducing percent of *Colletotrichum capsici*, a seedborne pathogen causing anthracnose disease in hot pepper seeds. Biomatriconditioning with clove leaf powder was better than matriconditioning plus fungicide in reducing infection level of *C. capsici*-infected hot pepper seeds, and improving seed viability and vigor for up to 24 weeks storage at ambient temperatures. In an experiment using bio-agents, the most effective seed treatment in reducing *C. capsici* contamination was biomatriconditioning with *Trichoderma harzianum* (83.7% reduction) followed by biomatriconditioning with *T. pseudokoningii* (82.3%), *Bacillus* sp. (79.8%), *Gliocladium* sp. (79.7%), and *Pseudomonas fluorescens* (78.3%). However, biopriming was better than biomatriconditioning in improving germination percentage and vigor index. In the field, biopriming with a mixture of *Bacillus polymixa* BG25 or *P. fluorescens* PG01 reduced anthracnose disease incidence from 81% in untreated infected seeds down to 9%, and improved plant growth, fruit yield, and seed quality of harvested seeds.

Keywords: anthracnose disease, bio-agent, biocontrol seed treatment, clove oil/powder, matriconditioning, rhizobacteria, seed vigor

INTRODUCTION

Anthracnose is a seedborne disease in hot pepper, caused by *Colletotrichum capsici*. The disease develops under high temperatures and wet conditions in the field, causing major damage and reducing yields of hot pepper. Symptoms of *C. capsici* infection are circular sunken spots on green and ripe fruits with pinkish or yellowish masses of glue-like spores accompanied by tiny black bristles (setae).

Seedborne pathogens in seeds generally either prevent germination or cause disease epidemics due to transmission of the pathogens. One infected seed may cause infection of many seedlings in a seedbed before transplanting. In some cases, however, infected seed may not exhibit reduce seed viability nor vigor. Hot pepper seeds highly infected by anthracnose may not show reductions in viability or vigor. Contaminated seeds germinate normally and carry the pathogen to the field during transplanting. Therefore, to obtain healthy seeds may require availability of effective seed treatments to eradicate seedborne pathogens. Removal of such pathogens should improve seed quality and plant stand.

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Matriconditioning is controlled seed hydration by moistened carriers with high water adsorptive matric forces (Khan et al., 1990; Khan, 1992). Bioprotectants and/or chemical pesticides can be used in conjunction with matriconditioning (Khan et al., 1992a, b). Ilyas and Sopian (2013) proposed the term 'biomatriconditioning' for the integration of bioprotectants into matriconditioning. Biomatriconditioning is a type of biological seed treatment using fungi or bacteria instead of using synthetic chemicals to control soil-borne and seed-borne pathogens.

Matriconditioning is also a suitable seed enhancement technique for hot pepper seeds (Ilyas, 1993, 2006; Ilyas et al., 2002). Combining matriconditioning with biopesticides e.g., clove (*Syzygium aromaticum*) oil or leaf powder, or biocontrol agents (e.g., rhizobacteria) may both reduce the infection levels of seedborne pathogens and improve seed viability and vigor.

Development of seed treatments using biopesticide and biocontrol agents has been investigated in our laboratories by several students as part of their thesis and dissertation (Suryani, 2003; Untari, 2003; Asie, 2004; Kumalasari, 2005; Sutariati, 2005). The objectives of the study were to evaluate effects of: (1) clove oil in hot pepper seed viability, vigor and *C. capsici*-infection level; (2) biopesticides on hot pepper seed storability; and (3) matriconditioning plus biocontrol agents on seed quality and seed health. Expected outputs were effective seed treatments to eradicate *C. capsici* infection in hot pepper seeds, and to improve seed health, seed viability and vigor, seedling stand establishment, and yield.

MATERIALS AND METHODS

Experiment 1. Effect of clove oil on seed viability, vigor, and *C. capsici* infection level

1. Seed materials and experimental design.

Seeds of hot pepper 'Hot Beauty', infected by *C. capsici* at 46% were used. Infected seeds were treated with either 0.06 or 0.1% clove oil, with or without matriconditioning, and there was an untreated control. Each treatment was repeated four times with 100 seeds per replicate. The experiment was a completely randomized design.

2. Matriconditioning procedure.

Seeds were matriconditioned in a 250 mL culture jar by mixing seeds, water and carrier of burned rice hull, which was homogenized and sieved using a 65 mesh screen. Ratio of seeds to carrier to water was 2:1:1 by weight. Clove oil (0.06 or 0.1%) was incorporated into matriconditioning. Seed mixtures were incubated at 22°C for four days and shaken for 1 min once a day. Seeds were washed with sterile water, wiped with tissues, and dried by forced air for 3 h after conditioning.

3. Seed viability and vigor.

Conditioned seeds were sown in a transparent box (25×20×5 cm) two-thirds full of burned rice hull. Fifty seeds were planted per box, and kept at ambient temperature. Germination was first recorded seven days after planting (dap) and final count 14 dap. Index of vigor was the first count germination. Speed of germination (SG) was the daily average of germinating seeds. Relative SG was SG/SG_{max}, where SG_{max} was the maximum germination/number of days of first count (14.2% day^{-1}).

4. *Colletotrichum capsici* infection level.

The level of *C. capsici* infection was determined using a deep-freezing blotter test. One hundred seeds (4×25) were placed on two layers of wetted sterile filter paper in Petri dishes (9-cm diam.), and incubated at room temperature for 12 h under NUV light and 12 h darkness, transferred to -20°C for 24 h, then back to room temperature for 12 days with a

day/night cylce of 12 h NUV light/12 h dark. Seeds were examined for *C. capsici* infection under a stereo binocular microscope 14 days after incubation (Watanabe, 2002). The ratio of infected to total hot pepper seeds was recorded.

Experiment 2. Effect of biopesticides on hot pepper seed quality and storability

1. Seed materials and experimental design.

Seeds of hot pepper 'Hot Beauty' infected by *C. capsici* at 25-50% were used. The initial seed viability and vigor were tested. Seeds were stored at 16°C, 70% RH for three months before the experiment. The experiment was arranged as a split plot with a completely randomized design. Main plots were storage period after seed treatment (five periods ranging from 0 to 24 weeks) and subplots were seed treatments [six treatments (matriconditioning alone, clove leaf, or dithane (mancozeb), with or without matriconditioning, and an untreated control)]. Each treatment was replicated four times with 100 seeds per replicate.

2. Preparation of powder biopesticide.

The biopesticide used was leaf of clove (*Syzygium aromaticum*), obtained from the Research Institute for Spices and Medicinal Crops, Bogor. Leaves were dried and homogenized with a grinder, sieved using 65 mesh screen, and stored in sealed flasks to prevent evaporation of volatile compounds.

3. Seed treatments and storage.

Seeds (1.3 g) were moistened with sterile water (0.2 mL) in a 250 mL culture jar and stirred for 5 min. Either 1.0% clove leaf powder (T2) or 0.2% Dithane M-45 (T4) was added to the moistened seeds and mixed until all seed surfaces were covered with powder. For T2, the ratio of seeds to clove leaf to water was 13:0.13:2 and for T4, the ratio of seeds to Dithane M-45 to water was 13:0.03:2. For matriconditioning (T1), seeds (1.3 g) were moistened with sterile water (0.65 mL) in transparent jars, burned rice hull powder (65 mesh, 0.65 g) was added and mixed until all seed surfaces were covered. The ratio of seed to carrier to water was 2:1:1 (Ilyas and Sudarsono, 2002). T3 and T5 were similar to T1, with the addition of clove leaf powder or Dithane M-45, respectively. All matriconditionings were conducted at 20-21°C and 60-70% RH for four days. T0 was an untreated control. Seeds were sieved to remove most of the attached materials, wiped with tissues, and dried by forced air for 3 h until reaching initial seed weight. Treated seeds were then packed in plastic bags, sealed and stored at ambient room (28-29°C and 60-70% RH). After storing for 0, 6, 12, 18, or 24 weeks, seed viability, vigor, and presence of *C. capsici* were evaluated.

4. Seed viability, vigor, and presence of *C. capsici*.

Seed viability, vigor, and presence of *C. capsici* were conducted using methods as described in Experiment 1. Germination percentage, normal seedling dry weight, and seedling growth rate were recorded.

Experiment 3. Effect of biopriming and biomatriconditioning

1. Seed materials and experimental design.

Seeds of hot pepper infected by *C. capsici* at 85% were used. Initial seed viability and vigor were tested. Seeds were stored at 16°C and 70% RH prior to the experiment. The experiment was a split plot with a completely randomized design. Main plots were biopriming or biomatriconditioning, and subplots were five biological agents (*Bacillus* sp., *Pseudomonas fluorescens*, *Trichoderma harzianum*, *T. pseudokoningii*, and *Gliocladium* sp.), and an untreated control. Each treatment was replicated four times with 100 seeds per

replicate.

2. Biopriming and biomatriconditioning procedures.

Fungal and bacterial biocontrol agents were prepared according to standard procedures for each agent. Seeds were primed or matriconditioned in 250 mL culture jars. Biopriming was done by mixing hot pepper seeds and a suspension of the biocontrol agent (2:1.5 by weight). Biomatriconditioning was done by mixing seeds, a suspension of the biocontrol agent, and carrier of burned rice hull (65 mesh). Ratio of seeds to carrier to agent was 2:1.5:1 (by weight). All seed mixtures were incubated at 22°C for four days, shaken for 1 min once a day, then washed with sterile water, wiped with tissues, and dried by forced air for 3 h after conditioning.

3. Seed viability, vigor and presence of *C. capsici*.

Seed viability, vigor and presence of *C. capsici* were evaluated using methods described in previous experiments. Percent germination and index of vigor were recorded.

4. Evaluation of treated seeds in the field.

In this experiment, only isolates of *Bacillus* sp. and *Pseudomonas* sp. were used. Seedlings derived from hot pepper seeds bioprimed with either *B. polymixa* BG25, *Pseudomonas fluorescens* PG01, a mixture of BG25+PG01, Dithane M-45, and untreated controls (both infected and healthy seeds) were grown in polyethylene bags in an open field, and evaluated up to maturity. Plant growth, disease incidence, fruit yield, and quality of harvested seed were recorded.

RESULTS AND DISCUSSION

Experiment 1. Effect of clove oil on seed viability, vigor, and *C. capsici* infection level

Clove oil at 0.06% or higher concentrations was highly inhibitory against *C. capsici* under in vitro conditions (data not shown). Clove oil (0.06 or 0.1%), with or without matriconditioning, significantly reduced infection levels of *C. capsici* in infected hot pepper seeds. However, only matriconditioning plus clove oil treatment (0.06 or 0.1%) improved index of vigor and relative speed of germination (Table 1). Percent germination was not affected by clove oil treatments, either with or without matriconditioning. These results were consistent with previous findings (Ilyas, 2006). Higher concentrations of clove oil were toxic to seeds, but clove oil toxicity was reduced when clove oil was integrated into matriconditioning.

Table 1. Effects of biopriming or biomatriconditioning with clove oil on level of contamination, percent of germination, relative speed of germination, and index of vigor of *Colletotrichum capsici*-infected hot pepper seeds.

Seed treatments[1]	Level of contamination (%)	Percent of germination (%)	Relative speed of germination (%)	Index of vigor (%)
Untreate	50 a[2]	69	57 b	5 c
Clove oil (0.06%)	6 b	80	69 a	31 b
Clove oil (0.1%)	4 b	66	50 b	8 c
Matric + Clove oil (0.06%)	3 b	76	71 a	47 a
Matric + Clove oil (0.1%)	3 b	80	74 a	49 a

[1]Matric – matriconditioning.

[2]Means in the same column suffixed with different letters are significantly different at α=5% according to DMRT.

Experiment 2. Effect of biopesticides on hot pepper seed quality and storability

At the begining of experiment, all treatments improved vigor and viability of evaluated seeds, and reduced infection levels of *C. capsici*-infected hot pepper seeds (Table 2). In general, *C. capsici*-infected hot pepper seeds treated with matriconditioning plus clove leaf powder were able to maintain seed physiological quality after 12 weeks of storage at ambient temperatures (Table 2). After 18-24 weeks of storage, seed viability (percent of germination, dry weight of normal seedling), and vigor (seedling growth rate) gradually decreased (Table 2) as seeds deteriorated. However, biomatriconditioned seeds showed better seed storability than that of matriconditioned plus Dithane M-45. Both seed treatment groups were equally effective in reducing *C. capsici* infection (Table 2).

Table 2. Effect of seed treatments and storage periods at ambient room temperature on seed viability and vigor, and *Colletotrichum capsici* infection level of hot pepper seeds.

Seed treatments[2]	Storage period (weeks)[1]				
	0	6	12	18	24
			Percent of germination (%)		
T_0	69.0 aD[3]	67.5 aE	66.5 aD	56.0 bC	49.5 cD
T_1	72.0 bC	75.5 abC	77.5 aB	72.0 bA	68.0 cAB
T_2	71.0 abC	73.5 aD	72.0 abC	71.5 abA	67.0 bB
T_3	79.0 aB	80.0 aB	**80.0 aA**	**73.0 bA**	**71.0 bA**
T_4	82.0 aA	81.5 aA	61.0 bF	34.0 cD	15.5 dE
T_5	79.0 bB	81.5 aA	65.0 cE	63.5 cB	55.0 dC
			Dry weight of normal seedlings (g)		
T_0	0.14 aE	0.13 aD	0.13 aC	0.10 bD	0.07 cD
T_1	0.15 bC	0.16 aB	0.17 aA	0.13 cAB	0.12 dB
T_2	0.15 bD	0.16 aC	0.15 bB	0.13 cB	0.11 dB
T_3	0.17 aAB	0.17 aA	**0.17 aA**	**0.13 bA**	**0.12 cA**
T_4	0.17 aA	0.17 aA	0.11 bE	0.06 cE	0.02 dE
T_5	0.17 aAB	0.17 aA	0.12 bD	0.12 bC	0.08 cC
			Seedling growth rate (mg)		
T_0	3.96 aB	3.98 aC	3.89 aC	3.55 bB	2.88 cB
T_1	4.22 aA	4.31 aA	4.25 aA	3.67 bA	3.41 cA
T_2	4.18 aA	4.23 aA	4.14 aB	3.63 bA	3.39 cA
T_3	4.23 aA	4.21 aA	4.14 aB	3.67 bA	3.39 cA
T_4	4.15 aA	4.16 aB	3.72 bE	3.22 cC	2.40 dC
T_5	4.22 aA	4.14 aB	3.80 bD	3.72 bA	2.96 cB
			C. capcisi infection level (%)		
T_0	42.5 bA	43.0 bA	43.5 abA	44.0 abA	49.0 aA
T_1	29.5 aB	22.0 bB	20.0 bcB	18.5 bcB	14.5 cB
T_2	21.0 aC	17.0 bC	12.5 bcC	10.5 bcC	9.0 cC
T_3	19.0 aD	15.5 abC	10.5 bC	9.0 bC	2.5 cD
T_4	11.5 aE	3.5 bD	3.0 bD	2.0 bD	0.5 bD
T_5	10.5 aE	4.5 abD	4.0 abD	2.5 bD	1.0 bD

[1] Seeds were packed in plastic bags, sealed and stored at 28-29°C and 60-70% RH.

[2] T0 = control; T1 = matriconditioning; T2 = clove leaf powder (CLP 1.0%); T3 = matriconditioning plus C (1.0%); T4 = Dithane M-45 (0.2%); T5 = matriconditioning plus Dithane M-45 (0.2%).

[3] For each recorded parameter, means suffixed with different uppercase letters in the same column or with lowercase letters in the same row are significantly different at α=5% according to DMRT.

In this study Dithane M-45 was used as a standard because Thind and Jhooty (1987) reported that Dithane M-45 at 0.2% was effective for controlling growth of *C. capsici* in chilli

pepper. In vitro experiments by Suryani (2003) also showed that Dithane M-45 was the most inhibitory fungicide against *C. capsici* colonies.

Experiment 3. Effects of biopriming and biomatriconditioning

Biomatriconditioning is the integration of a biocontrol agent in matriconditioning, while biopriming is the integration of a biocontrol agent in priming. Compared to untreated controls, both biopriming and biomatriconditioning were effective in reducing *C. capsici* infection in hot pepper seeds. However, biomatriconditioning was more effective for that purpose than biopriming. Results showed that all evaluated rhizobacteria in combination with matriconditioning were effective in reducing *C. capsici* (Table 3).

Table 3. Effects of biopriming or biomatriconditioning applied on *Colletotrichum capsici*-infected hot pepper seeds on percent of *C. capsici* infection.

Biocontrol agents	Bio-priming[1]			Bio-matriconditioning		
	Pre-	Post	Reduction	Pre-	Post	Reduction
	%					
Untreated	85	36	49	85	28	57
Bacillus sp.	85	6	79	85	5	80
P. fluorescens	85	20	65	85	7	78
T. harzianum	85	9	76	85	1	84
T. pseudokoningii	85	13	72	85	3	82
Gliocladium sp.	85	13	72	85	5	80

[1] Biopriming was conducted by soaking seeds (2 g) for 24 h in suspension of the respective biological agent (1.5 mL) at 26°C. Biomatriconditioning was conducted by using 2:1:1.5 ratio of seeds to carrier (burned rice hull 210 µ) to biological agent. Reductions were calculated using equation [(Pre-Post)/Pre]×100%.

Biopriming treatments were generally more effective than biomatriconditioning for increasing seed viability and vigor of *C. capsici*-infected hot pepper seeds (Table 4). Biopriming with *Bacillus* spp., *T. harzianum*, or *T. pseudokoningii* increased germination percent relative to the untreated control, while other biopriming treatments were not different from the control. However, biomatriconditioning either reduced or maintained germination percentage at the same level as in untreated controls (Table 4). These results confirm a previous report about positive effects of biopriming on percent of germination (Ilyas, 2006).

Table 4. Effects of biopriming or biomatriconditioning applied on *Colletotrichum capsici*-infected hot pepper seeds on percent of germination and index of vigor.

Biological agents	Germination percentage (%)		Index vigor (%)	
	Biopriming	Biomatriconditioning	Biopriming	Biomatriconditioning
Untreated	71 bA[1]	65 aB	56 dA	54 abB
Bacillus sp.	79 aA	60 abB	72 bcA	58 aB
P. fluorescens	72 bA	57 bB	67 cA	49 bcB
T. harzianum	78 aA	60 abB	81 aA	56 aB
T. pseudokoningii	78 aA	61 abB	75 abA	54 abB
Gliocladium sp.	67 bA	51 cB	70 bcA	43 cB

[1] Means in the same rows suffixed with different uppercase letters or the same column with different lowercase letters are significantly different at α=5% according to DMRT.

The use of rhizobacteria as seed treatments to control soil-borne and seed-borne pathogens is preferable to the use of synthetic chemicals because of human and environmental safety concerns and phytotoxicity problems associated with excess use of

pesticides. Biological seed treatments offer the potential for protecting treated seedlings not only at the seed or seedling stage but throughout their life cycle (Copeland and McDonald, 2001).

The application of biopriming in *C. capsici*-infected hot pepper seeds resulted in improved fruit yields and decreased anthracnose infection in field evaluations (Table 5). Biopriming with a combination of *B. polymixa* BG25 and *P. fluorescens* PG01 was the best seed treatment and resulted in significantly higher fruit yields. Anthracnose incidence was also the lowest in the plant population originating from seed treated with priming in combination with *B. polymixa* BG25 and *P. fluorescens* PG01. This treatment also increased germination and vigor index of seeds harvested from treated plants.

Table 5. Effects of biopriming applied on *Colletotrichum capsici*-infected hot pepper seeds on plant growth, fruit yield, disease incidence, and quality of harvested seeds.

Seed treatments[1]	Primary branch	Roots length (cm)	Fruit yield	Disease incidence (%)	Germination (%)	Vigor index (%)
BG25	4.7 b[2]	31.2 a	23 b	12 c	75 a	33 a
PG01	4.8 ab	31.6 a	24 b	12 c	75 a	32 a
BG25+PG01	5.2 a	32.4 a	27 a	9 d	78 a	37 a
Dithane M-45	3.6 c	24.9 b	15 c	60 b	65 b	22 b
Infected seed	3.4 c	19.9 c	10 d	81 a	56 c	18 b
Healthy Seed	3.6 c	24.8 b	15 c	62 b	62 b	21 b

[1] BG25 (*Bacillus polymixa* BG25), PG01 (*Pseudomonas fluorescens* PG01). Biopriming with rhizobacteria was conducted by soaking seeds (1 g) for 24 h in suspension of the isolate (50 mL) at 26°C. Subsequently, seeds were dried for 1 h in a laminar air flow cabinet. Seed treatments with Dithane M-45 were done similarly but seeds were soaked in the fungicide solution.

[2] Means in a column suffixed with different letters are significantly different at α=5% according to DMRT.

CONCLUSIONS

Biomatriconditioning using burned rice hull (65 mesh) with clove oil (0.06 or 0.1%) improved physiological seed quality and reduced *C. capsici* contamination level of hot pepper seeds. Biomatriconditioning with clove leaf powder was better for reducing *C. capsici* infection level of hot pepper seeds than matriconditioning plus fungicide. This treatment also improved storability of infected hot pepper seeds. Biomatriconditioning with *T. harzianum* or *T. pseudokoningii* were the best treatments to reduce *C. capsici* contamination level. However, biopriming was better than biomatriconditioning in improving percent germination and index of vigor. Biopriming with a mixture of *B. polymixa* BG25 and *P. fluorescens* PG01 reduced anthracnose disease incidence, improved plant growth, fruit yield, and seed quality of harvested seeds.

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