British Microbiology Research Journal
4(7): 737-748, 2014

SCIENCEDOMAIN *international*
www.sciencedomain.org

Role of Different Additives on Survival of *Serratia plymuthica* HRO-C48 on Oilseed Rape Seeds and Control of *Phoma lingam*

Ruba Abuamsha[1], Mazen Salman[2*] and Ralf-Udo Ehlers[3]

[1]Department of Biotechnology, National Agriculture Research Center (NARC) Jenin, West Bank, Palestine.
[2]Technical and Applied Research Center (TARC), Palestine Technical University-Kadoorie, Tulkarm- West Bank, Palestine.
[3]Department of Biotechnology and Biological Control, Institute for Phytopathology, Christian Albrecht University of Kiel, Germany.

Authors' contributions

This work was carried out in collaboration between all authors. Author RA designed the study, performed the experimental work wrote the protocol, and wrote the first draft of the manuscript. Author MS performed statistical analysis and managed the analyses of the study. Author RUE supervised the work. All authors read and approved the final manuscript.

Original Research Article

Received 11th December 2013
Accepted 17th January 2014
Published 21st March 2014

ABSTRACT

For optimum inhibition of plant pathogens, biocontrol agents must be maintained at higher density and survive for a long time in the plant system. Biopriming of seeds with bacteria in the presence of different additives provide a promising technique that might improve the efficacy of biocontrol agents and their application. The aim of this work was to test different additives and stickers on the activity of *Serratia plymuthica* in oilseed rape in controlling *Phoma lingam*. Seeds were soaked in bacterial suspensions (log_{10} 11 CFU ml^{-1}) containing one of the following Stickers and additives in a ratio of 1:1:1 (w:v:v): Sodium alginate, Dextran T 40, Polyvinyl alcohol, Methylcellulose, Gum Arabic, Raffinose, Tween 20, and Paraffin oil. Seeds were stored either at room temperature or at 4°C. Number of bacteria inside the seeds was monitored over a period of 12 months. The effect of additives and stickers on bacterial efficacy in controlling *Phoma lingam* was evaluated in pot experiments. Number of bacterial cells inside the seeds was significantly higher in the seeds coated with GA+$MgSO_4$, RF+MC and PA (log_{10} 7.5 ± 0.2, log_{10} 7.5 ± 0.2 and log_{10}

**Corresponding author: Email: salman_mazen@daad-alumni.de;*

7.4 ± 0.4, respectively). Interestingly, bacterial concentration in seeds stored at 4°C was higher than that in seeds stored at 20°C. Moreover, after storage for 8 months, *Serratia plymuthica* was able to control the black leg disease. Our results showed that some additives and stickers prolonged the shelf life of bacteria inside the seeds and improved the efficacy of bacteria in controlling the disease.

Keywords: Biological control; bacteria; plant pathogens; additives; stickers.

1. INTRODUCTION

Biological seed treatments to incorporate biocontrol agents (BCAs) using bio-priming and coating procedures provide an economical delivery system of BCAs compared to other field application systems [1,2,3]. Small amount of inoculum is needed for establishment of BCAs on germinated seeds and rhizosphere [4]. In addition to that, BCAs cells inside the seeds are protected from adverse environmental conditions [5]. However, lack of consistent effectiveness, storage stability and shelf life of products being applied to the seed, are limiting factors for commercial development and application of BCAs products [6,7,8]. Moreover, the products should be safe, effective over a wide range of field conditions and not be deleterious to the seed [9].

For effective suppression of plant disease, it is important to develop BCA products that maintain high density of BCA inoculant lasting with a high survival rate during storage not less than 24 months at 4°C [10]. Most BCAs have a threshold level needed for biocontrol above $\log_{10}$ 5 CFU g^{-1} seed, root, or soil [4,11]. For this reason, it is necessary to improve the performance and reliability of BCA products to be delivered and established in the potential infection zone of the host plant or in the rhizosphere and to have a consistent efficacy under different field conditions at commercially feasible rates.

The development of such product involves optimization of BCA delivery, by selecting compatible amendments (additives or adjuvants) that promote optimum, stable and effective BCA density during storage to stabilize and protect the BCA following sowing from desiccation and other adverse environmental conditions as well as to permit rapid establishment of the BCA in the rhizosphere and spermosphere [11,12,13].

In recent years several types of amendments that could be utilized in the biological seed treatment process have been used [14]. The objectives of this study were to develop and optimize seed bio-priming and coating treatment of *Serratia plymuthica* on oilseed rape (OSR) seeds, through screening for suitable additives. The effect of different additives on the shelf life of *S. plymuthica* on OSR seeds and seed germination was investigated.

2. MATERIALS AND METHODS

2.1 Bacterial Inoculums

A spontaneous rifampicin-resistant isolate of *S. plymuthica* HRO-C48 was used to facilitate the identification of the bacterium in the bio-primed seeds and OSR-rhizosphere. The bacterium was grown in 250 ml Erlenmeyer flasks containing 50 ml TSB (30 g l^{-1} tryptic soy broth) liquid medium supplemented with 150 µg l^{-1} rifampicin (bioMérieux Deutschland GmbH, Nürtingen) on a rotary shaker (150 rpm) in the dark for 48 h at 28°C. Bacterial

concentration was determined spectrophotometrically at optical density of 600 nm (OD_{600}) and adjusted to a final concentration of $\log_{10}$ 11 CFU ml^{-1} in sterile 0.85% NaCl [15].

2.2 Fungal Strain and Culture Conditions

Phoma lingam was maintained on V-8 media (V-8 juice 200 ml, $CaCO_3$ 0.75 g, Agar 15 g and 800 ml of distilled H_2O). Pycnidiospores of *P. lingam* were prepared by growing the fungus in 250 ml Erlenmeyer containing 50 ml V-8 liquid medium for 14 days at 24°C on a rotary shaker at 180 rpm. The suspension was filtered through sterile gauze (Paul Hartmann, Wiener Neudorf, Germany) and the spore concentration was adjusted to $\log_{10}$ 7 spores ml^{-1} using haemocytometer.

2.3 Preparation of Sticker and Additive Solutions

Stickers and additives used in this study were: Sodium alginate (Na-A) (Hahn), Dextran T 40 (DX) (Roth), Polyvinyl alcohol (PA) (Aldrich-Sigma), Methylcellulose (MC) (Sigma), Gum Arabic (GA) (Sigma), Raffinose (RF) (Sigma), Tween 20 (TW) (Roth), and Paraffin oil (PO) (Merk). PA, DX, and MC were tested at concentrations of 2% solutions (w/v). RF and Na-A, were tested at 1%. TW was tested at 0.01% and GA at 40%. All stickers and additives were dissolved in water except for Raffinose which was dissolved in 0.85% NaCl.

2.4 Coating and Bio-priming of Oilseed Rape Seeds

Oilseed rape seeds cultivar Talent used in this study were obtained from Norddeutsche Pflanzenzucht, Hans-Georg Lembke KG, Hohenlieth, Germany. The cultivar is moderately resistant to blackleg disease with a rating of 5 in a scale from 1 (low) to 9 (high resistance) [16]. Seeds priming with the bacteria was done as mentioned in Abuamsha et al. [17].

Seeds were soaked in bacterial suspensions ($\log_{10}$ 11 CFU ml^{-1}) grown in TSB media in Erlenmeyer flasks on a rotary shaker set at 150 rpm for 4 h at room temperature. Different additive mixtures were then added to the flasks for 2 h. Each mixture contained 1 g seeds, 1 ml bacterial suspension and 1 ml additive solution in a ratio of 1:1:1 (w:v:v). For control experiment, seeds were immersed in above mentioned suspensions and in sterile distilled water. The seeds were then air-dried on filter paper over night in growth chamber at 28°C. The bio-primed seeds were divided into two sets and stored either at 4 or 25°C in petri-dishes sealed with parafilm.

2.5 Viability Test

After bio priming of seeds, three samples (10 seeds each) were selected randomly and ground in 1 ml of 0.85% NaCl solution using sterile mortar and pestle. Serial dilutions were plated on TSA media supplemented with rifampicin (100 µl ml^{-1}). After incubation at 28°C in the dark for 48 h, number of bacteria per seed was determined. Long term survival of antagonistic bacteria on OSR seeds was assessed monthly over a period of 14 months.

2.6 Germination Assay

The viability of seed treated with different additives and stickers with or without bacteria was tested in 90 mm-diameter plates. 50 seeds from each treatment were spread on Whattman # 1 filter paper wetted with tap water. The plates were then incubated in dark at room

temperature 20 ± 2°C for three days. Germinated seeds were then counted and the percent of germination was calculated. The experiment was done in triplicates and repeated three times.

2.7 Growth Chamber Pot Experiments

Growth chamber pot experiments were conducted to determine the effect of different bio priming treatments against *P. lingam* as mentioned in Hammoudi et al. [18]. For this, 10 seeds from each treatment were selected randomly at different intervals (0, 4, 8 and 12 months), and sown in 9-cm diameter plastic pots filled with soil (Einheitserdewerk, Uetersen, Germany). The pots were incubated at a temperature of 21/16°C, 80/70% relative humidity (day/night) and 16/8 h light/dark regime.10 days after sowing the cotyledons were wounded in the center with a sterile needle and 10 µl droplets of *P. lingam* pycnidiospore suspension (log_{10} 7 spores ml^{-1}) were deposited onto each wound. Sterile water was used for control treatments. The inoculated plants were covered with plastic sheets to maintain high humidity for 24 h. Every two days, all leaves except the inoculated cotyledons we removed. After 14 day post inoculation, disease index (DI) was determined according to lesions sizes around the wound sites (width x length). Percent of healthy plants was calculated as well. Symptoms were classified on a scale of 0 - 6 (0 = no symptoms; 1 = lesion size < 5 mm^2; 2 = 5 – 10 mm^2; 3 = 11 – 15 mm^2; 4 = 16 – 20 mm^2; 5 = 21 – 30 mm^2; 6 = lesion size > 30 mm^2). The disease index was calculated based on 10 plants per tray as DI = [(n0 x 0) + (n1 x 1) + … + (n6 x 6) / (N x 6)] x 100, where n0 – n6 = the number of plants belonging to classes 0 – 6, and N = the total number of plants [19]. The mean DI for each treatment was based on the DI of three trays each with ten pots (plants). Experiments were conducted twice.

2.8 Statistical Analysis

Statistical analysis was done using XlStat program (Adinosoft). Data on CFU were log_{10} transferred and significant differences were computed using ANOVA after Tukeys HSD test at $P< 0.01$.

3. RESULTS

3.1 Enumeration of Antagonistic Bacteria on Coated-Bio-Primed Seed

The initial bacterial concentration on OSR seeds (determined immediately after seed bio priming) was more than the effective level for disease suppression (log_{10} 5 CFU g^{-1} seed) determined by Raaijmakers and Weller (1998) [20] (Fig. 1). Incorporation of GA+$MgSO_4$, RF+MC and PA in the bio-priming medium resulted in a significantly ($F = 402.7$, $df = 10, 98$, $p < 0.0001$) greater CFUs per seed of log_{10} 7.5 ± 0.2, log_{10} 7.5 ± 0.2 and log_{10} 7.4 ± 0.4, respectively. The lowest CFU $seed^{-1}$ (log_{10} 5.8 ± 0.3 and log_{10} 5.0 ± 0.4 CFU $seed^{-1}$) was recorded in seeds treated with Na-A and PO, respectively. In the control treatments (seeds bio primed with water), the number of *S. plyumthica* cells was log_{10} 6.6 ± 0.3 CFU $seed^{-1}$. Data in Fig. 1 shows also that in all treatments, except for Na-A and PO, the number of *S. plyumthica* cells was higher than that of the control treatment.

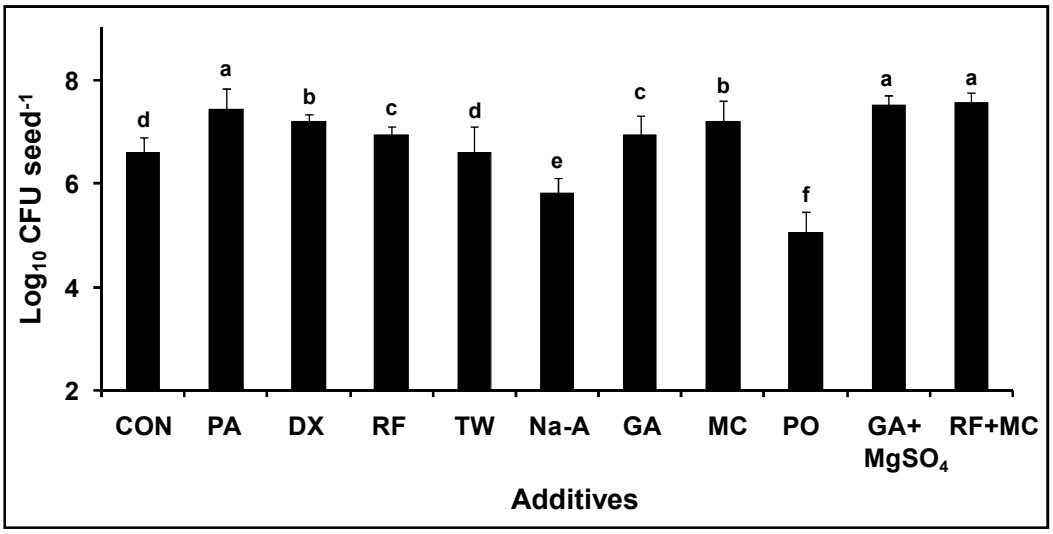

Fig. 1. Influence of different additives and stickers on the cell density of *S. plymuthica* inside the seeds. Seeds were soaked in bacterial suspension of (log_{10}11 CFU ml^{-1}) in TSB media first for 4 h followed by addition of 1 ml different solutions for 2 h. Seeds were agitated on a rotary shaker 150 rpm. Bacteria were re-isolated and cell numbers were counted. Same letters above bars symbolized no statistically significant differences at P ≤ 0.01. Error bars indicate standard deviations

3.2 Survival and Viability Test of *S. plymuthica* on Bio-Primed Seeds during Storage

Survival of *S. plymuthica* on the seeds stored at 4°C was significantly higher (F = 275.1, df = 27, 2771, p < 0.0001) than that at 20°C (Fig. 2). Storage of seeds at room temperature resulted in a pronounced reduction in bacterial counts on OSR seeds. The decline differed significantly between different additives (F = 423.0, df = 23, 1385, p < 0.0001). The average reduction was 94.1% after 8 months of storage at 20°C and 91.2% after 13 months at 4°C (Fig. 2). In term of the behavior during storage at 20°C, with the exception of RF+MC treatment, none of the additives showed a positive detrimental effects to the bacterial counts on seeds, as bacterial counts from seeds treated with the stickers did not differ from bacterial counts from seeds treated with water. While the opposite were detected when treated seeds stored at 4°C, all treatment performed well and enhanced the shelf-life of *S. plymuthica* compared with the control. At 4°C, different formulated additives were able to maintain effective density of *S. plymuthica* on seeds above the effective limit for disease suppression which is log10 5 CFU $seed^{-1}$ for 9 months in PA, DX and RF+MC, after that 47.9%, 49.2% and 57.5% reduction in the CFUs on the seeds from the initial count below the effective limit, respectively. GA+MgSO4 can support the effective CFUs on seeds for 8 months and MC, for 7 months, GA, for 6 months, water (CON), RF, and TW for 4 months and Na-A, PO for 2 months, after which 42.0% (GA+$MgSO_4$), 34.2% (MC), 47.3% (GA), 38.5% (CON), 42.9% (RF), 50.0% (TW), 27.2% (Na-A) and 7.9% (PO) reduction in CFUs below the effective limit occurred. The inoculums survival on seeds stored at 20°C was markedly lower with loss in cell density from the initial density at zero time, below the effective limit after one month by 40.3%, 46.1% and 34.3% in CON, Na-A and PO treatment, respectively. RF+MC support the effective density for nearly 6 months at room temperature

and 44.8% reduction below this level occurred next month. All other treatments retained the effective density of *S. plymuthica* on seeds for approximately 3 months with a reduction below the effective level next month by 47.4% (PA), 48.0% (DX), 38.8% (RF), 36.8% (TW), 38.8% (MC) and 42.7% (GA+MgSO4).

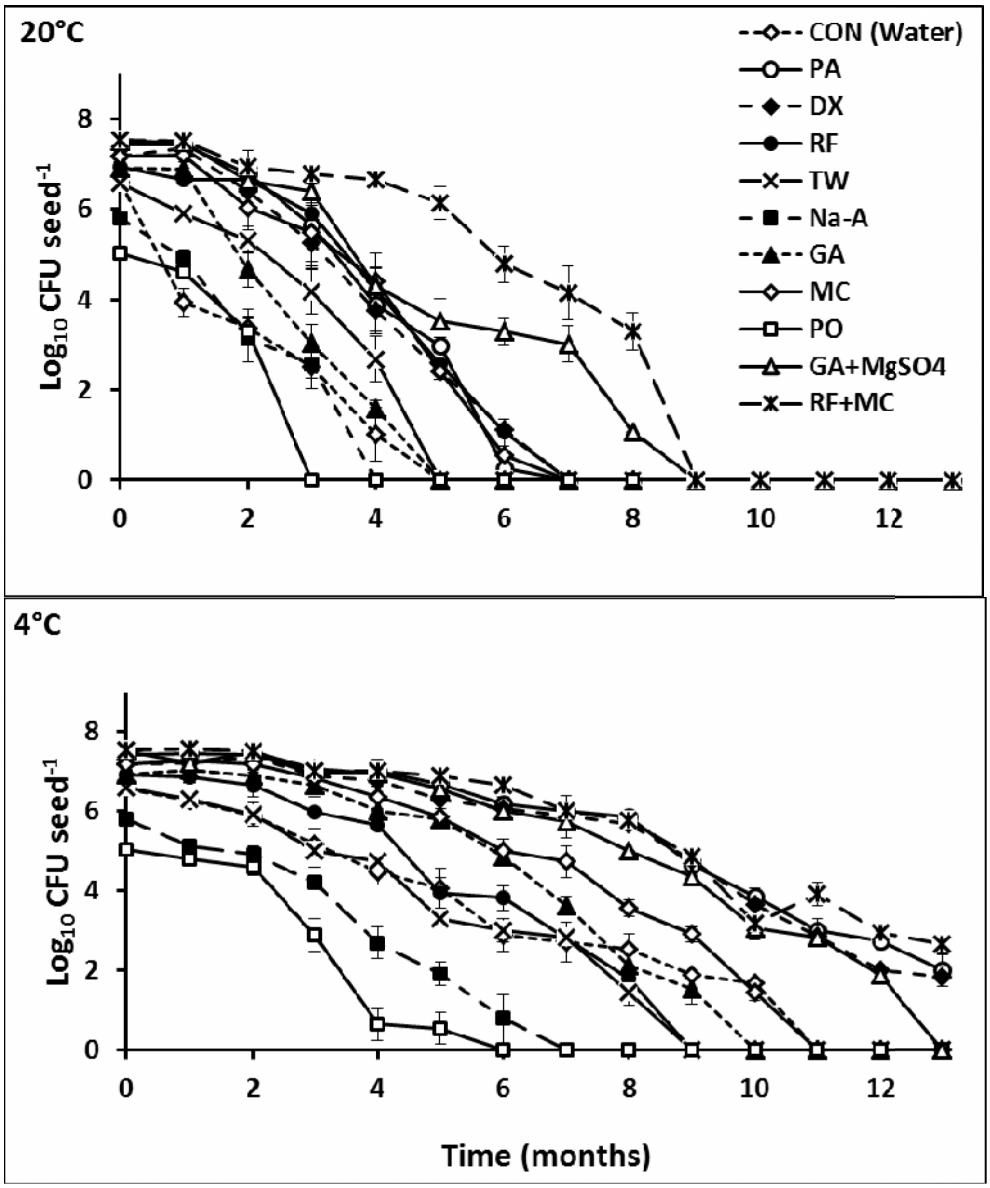

Fig. 2. Influence of different additives and stickers on the stability of a maximum number of CFU of *S. plymuthica* inside OSR seeds during storage at 4°C and at 20°C 1 g seeds were soaked in 1 ml bacterial suspension of (log_{10} 11 CFU ml^{-1}) in TSB media first for 4 h followed by addition of 1 ml different solutions for 2 h. Seeds were agitated on a rotary shaker 150 rpm. Bacteria were re-isolated monthly and cell numbers were counted. Error bars indicate standard deviations

Table 1. The effect of different additives and stickers with *S. plymuthica* and without (control) on the ability of OSR seed germination after storage at 4°C in the 1st month (Zero time), 4 months, 8 months and 12 months. 1 g seeds were soaked in 1 ml bacterial suspension of (log_{10} 11 CFU ml^{-1}) in TSB media first for 4 h followed by addition of 1 ml different solutions for 2 h (in *S. plymuthica* treatment) while for the treatment without bacteria 1 g seeds soaked in 1 ml different solutions for 2 h. Seeds were agitated on a rotary shaker 150 rpm. Data are represented as % of germination. A significant increase in the percent of germination in seeds primed with *S. plymuthica* compared with non primed seeds. Mean values in the same column followed by the same letter were not significantly different at P ≤ 0.01. Data are represented as means of % of germination ± SD.

Treatment	1st Month		4 Months		8 Months		12 Months	
	Control	*S. plymuthica*	Control	*S. plymuthica*	Control	*S. plymuthica*	Control	*S. plymuthica*
RF+MC	88.0 ± 0.7^{ab}	94.4 ± 2.0^{a}	86.0 ± 6.4^{ab}	92.4 ± 3.4^{ab}	84.2 ± 1.0^{bc}	92.4 ± 1.5^{a}	85.8 ± 4.7^{a}	89.1 ± 4.1^{ab}
PA	93.3 ± 2.3^{ab}	96.7 ± 2.0^{a}	93.3 ± 0.7^{a}	97.6 ± 2.3^{a}	91.8 ± 0.4^{a}	95.6 ± 0.4^{a}	86.2 ± 3.7^{a}	90.0 ± 1.3^{ab}
DX	91.1 ± 1.5^{ab}	95.8 ± 3.0^{a}	92.2 ± 2.1^{a}	98.0 ± 1.8^{a}	86.4 ± 3.7^{ab}	93.1 ± 3.8^{a}	81.6 ± 1.5^{a}	85.8 ± 1.5^{ab}
TSB	91.6 ± 3.7^{ab}	96.2 ± 1.0^{a}	93.6 ± 2.0^{a}	97.6 ± 1.0^{a}	89.3 ± 2.0^{ab}	93.6 ± 1.4^{a}	85.6 ± 4.5^{a}	91.8 ± 4.7^{a}
GA+$MgSO_4$	86.7 ± 7.0^{b}	92.2 ± 3.2^{ab}	88.7 ±1.2^{a}	93.3 ±2.0^{ab}	84.4 ± 2.7^{b}	92.2 ± 3.3^{a}	82.2 ± 0.8^{a}	82.0 ± 2.9^{b}
RF	94.4 ± 1.4^{a}	97.6 ± 2.7^{a}	93.1 ± 2.1^{a}	97.1 ± 3.9^{a}	85.8 ± 3.2^{ab}	91.8 ± 2.7^{a}	85.3 ± 3.7^{a}	85.1 ± 3.4^{ab}
MC	77.3 ± 3.5^{c}	84.0 ± 3.7^{c}	72.7 ± 2.0^{cd}	79.8 ± 2.3^{d}	61.6 ± 2.0^{e}	70.2 ± 7.1^{cd}	62.0 ± 5.5^{b}	62.4 ± 2.1^{cd}
GA	73.1 ± 4.5^{c}	83.8 ± 2.1^{c}	71.8 ± 6.2^{cd}	83.6 ± 2.7^{cd}	72.9 ± 7.0^{d}	73.1 ± 6.4^{bc}	61.1 ± 1.0^{b}	64.0 ± 2.4^{cd}
TW	78.0 ± 3.5^{c}	86.2 ± 5.6^{bc}	70.4 ± 9.3^{d}	80.4 ± 9.3^{d}	73.6 ± 6.2^{d}	71.8 ± 1.4^{cd}	62.4 ± 4.7^{b}	62.4 ± 4.7^{cd}
Na-A	89.1 ± 2.1^{ab}	94.0 ± 1.2^{a}	79.3 ± 4.4^{bc}	88.7 ± 1.8^{bc}	77.6 ± 1.7^{cd}	79.3 ± 2.0^{b}	63.3 ± 3.3^{b}	66.7 ± 0.0^{c}
Water	90.2 ± 3.4^{ab}	93.6 ± 0.4^{a}	88.9 ± 4.2^{a}	94.4 ± 3.0^{ab}	90.0 ± 0.0^{ab}	94.7 ± 1.2^{a}	83.1 ± 2.0^{a}	88.4 ± 3.7^{ab}
PO	64.9 ± 1.4^{d}	75.3 ± 4.7^{d}	57.1 ± 3.3^{e}	70.4 ± 1.5^{e}	64.0 ± 4.4^{e}	65.8 ± 4.3^{d}	52.7 ± 5.8^{c}	57.6 ± 2.3^{d}
Average ± SD	84.8 ± 9.8^{B}	90.8 ± 7.4^{A}	82.3 ± 12.2^{B}	89.4 ± 9.3^{A}	80.1 ± 10.4^{B}	84.5 ± 11.6^{A}	74.3 ± 12.8^{B}	77.1 ± 13.3^{A}

Table 2. Effect of *S. plymuthica* HRO-C48 Rifr combined (+) with different additives and stickers that stored at 4°C in the 1st month (Zero time), 4 months, 8 months and 12 months on the development of blackleg disease on OSR; Talent cultivars treated with *P. lingam* suspension (log_{10} 7 spores ml^{-1}, 10 µl $cotyledon^{-1}$). 1 g seeds were soaked in 1 ml bacterial suspension of (log_{10} 11 CFU ml^{-1}) in TSB media first for 4 h followed by addition of 1 ml different solutions for 2 h (+). Seeds were agitated on a rotary shaker 150 rpm. Data are calculated as percent reduction of control and % of healthy plants. Mean values of percent reduction of control in the same column followed by different letters are significantly different after Tukeys HSD test at P ≤ 0.01. Data represent mean percent healthy plants and percent reduction of control of DI ± standard deviation (SD).

Treatment	Reduction in DI (%)				% Healthy Plants			
	1st Month	4 Months	8 Months	12 Months	1st Month	4 Months	8 Months	12 Months
RF+MC	77.9 ± 1.7^{a}	72.7 ± 1.4^{a}	69.6 ± 2.5^{a}	58.7 ± 5.4^{a}	88.9 ± 0.9^{a}	86.7 ± 1.1^{a}	85.0 ± 1.1^{a}	80.6 ± 2.0^{a}
PA	74.1 ± 1.8^{ab}	65.6 ± 4.1^{ab}	57.7 ± 3.5^{bc}	40.1 ± 4.9^{b}	87.2 ± 1.4^{ab}	82.8 ± 2.3^{ab}	79.4 ± 1.4^{bc}	71.9 ± 1.6^{b}
DX	72.7 ± 4.0^{ab}	65.7 ± 1.6^{ab}	60.8 ± 3.5^{ab}	37.3 ± 7.1^{b}	86.4 ± 1.6^{ab}	83.1 ± 1.3^{ab}	80.6 ± 1.4^{ab}	70.6 ± 3.3^{b}
TSB	72.7 ± 3.6^{ab}	66.1 ± 2.8^{ab}	63.6 ± 3.3^{ab}	41.1 ± 2.7^{b}	86.7 ± 2.1^{ab}	83.3 ± 1.5^{ab}	81.7 ± 2.1^{ab}	72.2 ± 0.9^{b}
GA+$MgSO_4$	67.9 ± 3.6^{bc}	58.6 ± 3.4^{bc}	50.5 ± 3.8^{c}	32.9 ± 2.4^{b}	83.6 ± 1.9^{bc}	80.0 ± 1.5^{bc}	75.8 ± 1.4^{c}	68.3 ± 1.1^{b}
RF	67.5 ± 3.6^{bc}	65.1 ± 2.2^{ab}	61.7 ± 4.2^{ab}	32.9 ± 5.8^{b}	83.6 ± 2.2^{bc}	83.3 ± 1.1^{ab}	81.4 ± 1.9^{ab}	67.8 ± 2.5^{b}
MC	60.4 ± 6.9^{c}	52.2 ± 3.6^{cd}	37.8 ± 7.1^{d}	9.81± 6.7^{c}	80.0 ± 3.5^{cd}	76.4 ± 1.9^{cd}	69.7 ± 3.9^{d}	56.9 ± 2.7^{c}
GA	53.2 ± 4.1^{de}	46.0 ± 4.2^{d}	25.4 ± 5.9^{e}	11.0 ± 4.0^{c}	75.8 ± 2.3^{de}	73.9 ± 2.5^{d}	64.2 ± 2.7^{e}	57.5 ± 1.7^{c}
TW	52.5 ± 4.2^{de}	45.5 ± 4.4^{d}	36.5 ± 3.1^{d}	11.7 ± 4.1^{c}	76.1 ± 2.3^{de}	73.1 ± 2.2^{d}	69.4 ± 2.3^{d}	58.3 ± 1.5^{c}
Na-A	45.8 ± 5.3^{e}	33.3 ± 4.0^{e}	16.2 ± 3.4^{ef}	4.6 ± 1.9^{c}	72.8 ± 2.7^{e}	67.2 ± 1.4^{e}	59.7 ± 1.9^{ef}	54.2 ± 1.7^{c}
Water	45.2 ± 1.8^{e}	21.3 ± 6.3^{f}	13.7 ± 2.7^{f}	2.9 ± 3.5^{c}	71.7 ± 1.1^{e}	61.1 ± 2.9^{f}	58.3 ± 1.1^{f}	54.4 ± 1.4^{c}
PO	14.3 ± 1.3^{f}	10.1 ± 1.9^{g}	3.4 ± 4.3^{g}	-9.3 ± 4.3^{d}	56.7 ± 1.5^{f}	55.6 ± 1.4^{g}	53.3 ± 2.1^{g}	47.8 ± 1.7^{d}
Average ± SD	58.7 ± 17.7^{A}	50.2 ± 19.4^{AB}	41.4 ± 22.0^{B}	22.8 ± 20.0^{C}	79.1 ± 9.1^{A}	75.5 ± 9.6^{AB}	71.6 ± 10.5^{B}	63.4 ± 9.6^{C}

3.3 Effect of Additives on Seed Germination

No effect on shape and size of OSR-seedlings was recorded in seeds treated with the different additives (Table 1). In seeds treated with water, the percent of seed germination at zero time, 4, 8 and 12 months after sowing were 84.8 ± 9.8 and 82.3 ± 12.2, 80.1 ± 10.4 and 74.3% ± 12.8, respectively. The germination percent of bio-primed seeds at zero and 4 months times were 90.8 ± 7.4 and 89.4% ± 9.3, respectively. After 8 and 12 months, the percent of germination was 84.5 ± 11.6 and 77.1% ± 13.3, respectively. In seeds treated only with additives, the percent of germination (94.4% ± 1.4) was significantly higher ($F = 43.9$, $df = 11, 107$, $p < 0.0001$) in the presence of raffinose (RF). The lowest germination percent was in seeds treated with paraffin oil (PO) (64.9% ± 1.4), Storage of bio primed seeds did not affect seed germination (90%) until 8 months.

3.4 Efficacy of Bio-priming With Different Additives on Blackleg Disease Incidence

Results in Table 2 indicate that, some additives were able to enhance the ability of *S. plymuthica* to reduce significantly blackleg disease.The average reduction of disease in bio primed seeds after 4 months was 58.7 ± 17.7, 41.4 ± 22.0 and 22.8% ± 20.0 after 4, 8 and 12 months, respectively. In addition to that, the percentage of healthy plants was 79.1% ± 9.1 75.5 ± 9.6, 71.6 ± 10.5 and 63.4% ± 9.6, respectively after 4, 8 and 12 months respectively. Bio priming with RF+MC, PA, DX and TSB was significantly higher ($F = 175.9$, $df = 11, 71$, $p < 0.0001$) in reducing disease incidence (70% reduction). The percent of healthy plants (80%) was also significantly higher ($F = 157.0$, $df = 11, 71$, $p < 0.0001$).

4. DISCUSSION

For commercial production of BCA, the product must have an adequate shelf life, good survival ability of the BCA in the soil and must be compatible with chemical seed treatment [21,22]. Consequently the improvement of seed bio-priming technique becomes important especially at commercial scale. Seed bio-priming of OSR-seeds with *S. plymuthica* led to effective control of blackleg disease caused by *P. lingam* [17]. In this study, seed bio-priming with *S. plymuthica* in the presence of different additives significantly improved the BCA survival on seeds, increased seed germination ability and decreased severity of blackleg disease. Adjuvants that stabilize and sustain the BCA and provide protection for the BCA from desiccation and other harsh environmental conditions, permit dispersal of the BCA in the rhizosphere and spermosphere and enhance their efficacy against diseases were investigated in this study. In addition to that, additives should not have a deleterious effect on the growth of BCAs. Cell density ($\log_{10}$ 7 CFU $seed^{-1}$) on the bio-primed seeds was significantly improved when *S. plymuthica* was applied in combination with RF+MC, GA+$MgSO_4$ and PA compared with bio-primed control (TSB with water), (Fig. 1). This improvement may be attributed to both chemical and physical nature of these additives because they contain carbon and considerable quantities of inorganic and organic nutrients [23]. These materials together with the seed moisture and root exudates might increase seed coat permeability, influence the pH, provide nutrients for BCA and enhance proliferation of BCA in and on the seed [1,24]. Moreover, they are considered as an effective stickers and biomass carrier (MC, GA and PA) that protect the seeds from the harsh conditions. MC, GA and polyvinyl derivatives are used as bulking agents and carriers, and they were suitable ingredient in producing formulations containing BCA. In addition to that, they are used for seed treatment to control leaf blight disease of bambara ground nut (*Vigna*

subterranea) [25]. With the exception of Na-A and PO, bio-priming and treatment of OSR seeds with *S. plymuthica* and different stickers and additives, a significant increase in the number of bacteria (log_{10} 6-8 CFU $seed^{-1}$) was recorded. The density of *S. plymuthica* (log_{10} 5-8 CFU $seed^{-1}$) at room temperature was constant for more than 3 months in the presence of PA, DX, RF and GA+$MgSO_4$ and 6 months in the presence of RF+MC. The ability of the bacteria *S. plymuthica* to survive for a long time in bio primed seeds might be due to the fact that the bacteria is protected inside the seeds form the adverse environmental conditions. In addition to that, the additives provide nutrition for the bacteria. Interestingly, in seeds treated with RF+MC, GA+$MgSO_4$, PA and DX, *S. plymuthica* at 4°C survived for more than 8 months.

Results of this work showed that OSR seeds bio priming with *S. plymuthica* and the different additives has no negative effect on germination ability of the seeds. In contrast to that, the percent of germination in the seeds treated with *S. plymuthica* and different additives was significantly increased. The viability of the stored treated seeds was consistent for more than 4 months. After 12 months of storage at 4°C, the percent of seed germination was 80% in seeds treated with *S. plymuthica* combined with one of the following additives: RF+MC, PA, the control (TSB with water), water, RF and DX. The ability of seeds to germinate decreased after storage. However, some additives can enhance and maintain the vitality of the seeds for a long time [26,25]. Similar results were reported by Jensen et al. (2004) [2], who found that, bio-primed seeds treated with *Clonostachys rosea* strain IK 726 for controlling seed borne Alternaria spp, were viable after 5 months of storage at 4°C. Comparing with the control, incorporation of some additives such as RF+MC, PA, DX, TSB, Na-A, and RF in bio-priming media enhances the germination of OSR seed. Several antagonistic BCAs effective against phoma stem canker have been previously developed and reported [27,28,29,30]. Recently, *S. plymuthica* was proved its efficacy against blackleg disease in different cultivars of OSR [17]. In this work, control of *P. lingam* was possible after seed bio priming and storage. However, control of the disease as well as healthy plants production was decreased after growing seeds which were stored for more than 8 months. Variations in disease control among different additives were detected. The highest control effect and production of healthy plants was recorded when RF+MC, PA and DX were used in bio-priming process. The superior effects of the additives in bio primed seeds were remained after 12 months of storage period at 4°C.

5. CONCLUSION

In conclusion, additive and stickers had no negative effect on bacterial efficacy against germination of the seeds. *S. plymuthica* was viable for at least 9 months at 4°C and 4 months at 20°C.

ACKNOWLEDGEMENTS

We would like to thank the German Academic Exchange Service (DAAD) for their financial support to the first author.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Wright B, Rowse HR, Whipps JM. Application of beneficial microorganisms to seeds during drum priming. Biocon Sci Technol. 2003;13:599–614.
2. Jensen B, Knudsen IMB, Madsen M, Jensen DF. Biopriming of infected carrot seed with an antagonist, *Clonostachys rosea*, selected for control of seed borne *Alternaria* spp. Phytopathology. 2004;94:551–560.
3. Pill WG, Collins CM, Goldberger B, Gregory N. Responses of non-primed or primed seeds of 'Marketmore 76' cucumber (*Cucumis sativus* L.) slurry coated with *Trichoderma* species to planting in growth media infested with *Pythium aphanidermatum*. Sci Hortic. 2009;121:54–62.
4. Bennett AJ, Whipps JM. Beneficial microorganism survival on seed, roots and in rhizosphere soil following application to seed during drum priming. Biol Control. 2008;44:349–361.
5. Müller H, Berg G. Impact of formulation procedures on the effect of the biocontrol agent *Serratia plymuthica* HRO-C48 on Verticillium wilt in oilseed rape. Biocontrol. 2008;53:905–916.
6. Mathre DE, Callan NW, Johnston RH, Miller JB, Schwend A. Factors influencing the control of *Pythium*-induced seed decay by seed treatment with *Pseudomonas aureofaciens* AB254. Crop Protect. 1994;13:301–307.
7. Mao W, Lumsden RD, Lewis JA, Hebbar PK. Seed treatment using pre-infiltration and biocontrol agents to reduce damping-off of corn caused by species of *Pythium* and *Fusarium.* Plant Dis. 1998;82:294–299.
8. Burgess DR, Keane PJ. Biological control of *Botrytis cinerea* on chickpea seed with *Trichoderma* spp. And *Gliocladium roseum*: indigenous versus non-indigenous isolates. Plant Pathol. 1997;46:910–918.
9. Paulitz TC. Population dynamics of biocontrol agents and pathogens in soils and rhizospheres. Eur J Plant Pathol. 2000;106:401–413.
10. Burgues HD, Jones KA. Trends in formulation of microorganisms and future research requirements. In: Burgues HD editor. Formulation of Microbial Pesticides Beneficial Microorganisms, Nematodes and Seed Treatments. London: Kluwer Academic 1998.
11. Raaijmakers JM, Leeman M, van Oorschot MMP, van der Sluis I, Schippers B, Bakker PAHM. Dose-response relationships in biological control of Fusarium wilt of radish by *Pseudomonas* spp. Phytopathology. 1995;85:1075–1081.
12. Schmidt CS, Lorenz D, Wolf GA, Jäger J. Biological control of grapevine dieback fungus Eutypalata II: influence of formulation additives and transposon mutagenesis on the antagonistic activity of *Bacillus subtilis* and *Erwinia herbicola*. J Phytopathol. 2001;149:437–445.
13. Spadaro D, Gullino ML. Improving the efficacy of biocontrol agents against soil borne pathogens. Crop Prot. 2005;24:601–613.
14. Wiyono S, Schulz DF, Wolf GA. Improvement of the formulation and antagonistic activity of *Pseudomonas fluorescens* B5 through selective additives in the pelleting process. Biol Control. 2008;46:348–357.
15. Taylor AG, Harman GE. Concepts and technologies of selected seed treatments. Annu Rev Phytopathol.1990;28:321–340.
16. Abuamsha R, Salman M and Ehlers RU. Differential resistance of oilseed rape cultivars (*Brassica napus* ssp. oleifera) to *Verticillium longisporum* infection is affected by rhizosphere colonization with antagonistic bacteria, *Serratia plymuthica* and *Pseudomonas chlororaphis*. Bio Control. 2011;56:101–112.
17. Rapool online: Available: http://www.rapool.de.

18. Abuamsha R, Salman M, Ehlers RU. Effect of seed priming with *Serratia plymuthica* and *Pseudomonas chlororaphis* to control *Leptosphaeria maculans* in different oilseed rape cultivars. Eur J Plant Pathol. 2011;130:287–295.
19. Hammoudi O, Salman M, Abuamsha R, Ehlers RU). Effectiveness of Bacterial and Fungal Isolates to Control *Phoma lingam* on Oilseed Rape *Brassica napus*. American Journal of Plant Sciences. 2012;3:773-779.
20. Borges AA, Cools HJ, Lucas JA. Menadione sodium bisulphate: a novel plant defence activator which enhances local and systemic resistance to infection by *Leptosphaeria maculans* in oilseed rape. Plant Pathol.2003;52:429–436.
21. Raaijmakers JM, Weller DM. Natural plant protection by 2,4-diacetylphloroglucinol-producing Pseudomonas spp.in take-all decline soils. Mol Plant Microb Interact. 1998;11:144–152.
22. Walker R, Rossall S, Asher MJC. Comparison of application methods to prolong the survival of potential biocontrol bacteria on stored sugar-beet seed. J Appl Microbiol. 2004;97:293–305.
23. Howell CR. Effect of seed quality and combination fungicide–*Trichoderma* spp. seed treatments on pre- and post emergence damping off in cotton. Phytopathology. 2007;97:66-71.
24. Taylor AG, Klein DE and Whitlow TH. SMP: Solid matrix priming of seeds. Sci. Hortic.1988;37:1-11.
25. Nelson EB. Microbial dynamics and interactions in the spermosphere. Annu Rev Phytopathol. 2004;42:271–309.
26. Pengnoo A, Wiwattanapattapee R, Chumthong A , Kanjanamaneesathian M. Bacterial antagonist as seed treatment to control leaf blight disease of bambara groundnut (*Vigna subterranea*). World J Microb Biot. 2006;22:9–14.
27. Bardin SD, Hung-Chang H. Efficacy of Stickers for Seed Treatment with Organic Matter or Microbial Agents for the Control of Damping-off of Sugar Beet. Plant Pathology Bulletin. 2003;12:19-26.
28. Chakraborty BN, Chakraborty, Basu K. Antagonism of *Erwinia herbicola* towards *Leptosphaeria maculans* causing blackleg disease of *Brassica napus*. Lett Appl Microbiol.1994;18:74–76.
29. Beatty PH, Jensen SE. *Paenibacillus polymyxa* produces fusaricidin-type antifungal antibiotics active against *Leptosphaeria maculans*, the causative agent of blackleg disease of canola. Can J Microbiol. 2002;48:159–169.
30. Hysek J, Vach M, Brozova J, Sychrova E, Civinova M, Edelnik J, Hruby J. The influence of the application of mineral fertilizers with the biopreparation suppressive (*Trichoderma harzianum*) on the health and the yield of different crops. Archives of Phytopathology and Plant Protection. 2002;35(2):115–124.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=469&id=8&aid=4081