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Standardization of the dosage of liquid formulation of *Bacillus amyloliquefaciens* and *Pseudomonas spp.* for sesame seed treatment and assessing the spermosphere colonization

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Abstract
A two days old liquid formulation of *B. amyloliquefaciens* containing 83.67 x 10^{-10} cfu / 0.1 ml was used as such in different quantities for treating one kg of sesame seed *i.e.* 10 ml/kg, 75 ml/kg, 100 ml/kg and 120 ml/kg of seeds and stored separately at room temperature for seventy five days. Then these treated seeds from each treatment were washed with known quantity of sterile distilled water individually and assessed the spermosphere population of *B. amyloliquefaciens* separately. The suspension collected from the seeds stored for seventy five days after treating with liquid formulation of *B. amyloliquefaciens* @ 10 ml/kg recorded 2.67 x 10^{-9} cfu / 0.5 ml suspension at 48 hours after plating. In another experimental study when the seeds were used ninety days after seed treatment for conducting roll towel method clearly revealed that the seed treated with liquid formulation of *Pseudomonas spp.* @ 20 ml/kg seed recorded highest shoot and root length of ten days old seedlings by recording 12.86 and 9.92 cm, respectively and the seeds treated with liquid formulation of *B. amyloliquefaciens* @ 20 ml/kg was found to record increased shoot and root length of seedlings by 12.42 cm and 9.34 cm respectively. These two treatments were statistically on par with each other.

Keywords: *Bacillus amyloliquefaciens*, *Pseudomonas spp.*, liquid formulation and sesame

Introduction
The major constraints that reduce the crop yield worldwide in oilseeds are plant pathogenic soil borne fungus *M. phaseolina* and leaf blight pathogen *A. sesami*. Cultivation of disease free crops in an ecofriendly approach is a great challenge being faced in recent days. Plant growth-promoting rhizobacteria (PGPR) is an excellent candidate in exerting the beneficial effects on plant growth which is often related with the increase of nutrient availability to host plant (Vessey, 2003) [18]. Bio-efficacy of talc-based formulation of PGPR isolates has already been demonstrated in different agriculture and horticulture crops against pests, diseases and nematodes under glasshouse and field conditions (Ramamoorthy *et al*., 2002; Bharathi *et al*., 2004; Jonathan *et al*., 2009; Saravanakumar *et al*., 2009) [13, 3, 5, 9]. However, talc based bio-inoculants were suffered from short shelf life and high contamination Shravani (2019) [16]. In this regard, liquid formulation of bio-agents was found to be an effective alternate to overcome these problems. Present study has been carried out with the objective of assessing the survival ability and establishment of *B. amyloliquefaciens* and *Pseudomonas spp.* in the spermosphere of sesame seeds when they were used in liquid formulation for seed treatment and also their effect on the growth of seedlings.

Materials and Methods
An experiment was conducted to standardize the dosage of liquid formulation of the antagonists *viz., Bacillus amyloliquefaciens and Pseudomonas spp.* for treating the sesame seeds against pathogens. *B. amyloliquefaciens and Pseudomonas spp.* were grown in Nutrient broth and King's B broth respectively under *in vitro* for two days. Then the population of *B. amyloliquefaciens* in the liquid formulation was recorded by following serial dilution technique upto 10^{-10} dilution level at 24 hrs and 48 hrs after plating. A plain broth was used to maintain control. Three replications were maintained for each dilution level.
A two days old liquid formulation of *B. amyloliquefaciens* containing 83.67 x10^{-10} cfu/ 0.1 ml was used as such in different quantities for treating one kg of sesame (variety CO 1) seeds *i.e.* 10 ml/kg, 75 ml/kg, 100 ml/kg and 120 ml/kg seed.

Then treated seeds were stored in the room temperature for 75 days in order to assess the survival and establishment of the *B. amyloliquefaciens* in the spermosphere. Five gram of treated seeds were collected from each treatment and transferred into sterile test tubes each containing 10 ml of sterile distilled water separately and each tubes were shaked well for 10 min and kept for 30 minutes. One ml of the suspensions was pipetted out from each treatment separately and serially diluted up to 10^{-9} by following serial dilution technique. Then from each dilution level, 0.5 ml was carefully pipetted out and poured into Petridish containing Nutrient agar medium under aseptic condition and rotated both clock and anti-clock wise direction to get an uniform thin smear of the bacterial culture over the medium. Each treatment was replicated three times and a control was also maintained. The bacterial colonies were counted 24 and 48 hrs after plating. Moreover, twenty five treated seeds were taken from each treatment of the above experiment separately and used for conducting germination test by roll towel method. Four replications were maintained for each treatment. The shoot and root length of ten days old seedlings were recorded as mentioned below.

1. Root length: In each treatment root length was measured in cm from the collar region to the tip of the primary root of the ten days old seedlings and the mean values were calculated separately.

2. Shoot length: The shoot length of seedlings in each treatment was measured in cm from the collar region to the growing tip of the shoot of the 10 days old seedlings and the mean values were calculated separately.

In another experiment, sesame (variety CO 1) seeds were treated with liquid formulation of *B. amyloliquefaciens* and *Pseudononas* spp. each at the rate of 10, 15, 20 and 25 ml/kg seed separately. Then a known quantity of seeds were treated with carbendazim @ 2 g / kg and all these treated seeds were stored at room temperature for 90 days (Mesterházy *et al*., 2011) [12]. A control was also maintained. Twenty five treated seeds were taken from each treatment separately and used for conducting germination test by roll towel method. Three replications were maintained for each treatment. The shoot and root length of ten days old seedlings were recorded as mentioned above.

Results and Discussion

In the two days old liquid formulation, the population of *B. amyloliquefaciens* was recorded as 42.67 x 10^{-10} cfu /0.1 ml and 83.67 x 10^{-10} cfu /0.1 ml at 24 hrs and 48 hrs after plating respectively (Table 1). The suspension or seed wash prepared from the seeds treated with liquid formulation of *B. amyloliquefaciens* @ 10 ml / kg and stored for 75 days at room temperature was found to record 2.67× 10^{-9} cfu / 0.5 ml suspension at 48 hrs after plating. Whereas the seeds treated with liquid formulation of *B. amyloliquefaciens* at the rate of 75 ml, 100 ml and 120 ml / kg seed recorded no bacterial colonies at 10^{-9} dilution level at 48 hrs after plating (Table 2). The results clearly indicates that treating the sesame seeds with low dose of liquid formulation of *B. amyloliquefaciens* was found to record the establishment and multiplication of this antagonist in the spermosphere far better than seeds treated with high doses of *B. amyloliquefaciens* (Table 2).

The sesame (variety CO 1) seeds treated with liquid formulation of *B. amyloliquefaciens*@ 10 ml / kg and stored for seventy five days was found to record high root (8.96 cm) and shoot length (11.76 cm) of ten days old seedlings. Moreover, when the seeds treated with liquid formulation of *B. amyloliquefaciens* @ 75 ml / kg recorded only 8.81 and 11.99 cm of root and shoot length respectively and these two treatments were found to be statistically on bar with each other. There was no increase in the root and shoot length of ten days old seedlings with corresponding to increase in the dosage of antagonist used for seed treatment (Table 3).

Ninety days after seed treatment, the seeds were drawn from the seed samples which individually treated with *B. amyloliquefaciens* and *Pseudomonas* spp. @ 10 ml, 15 ml, 20 ml and 25 ml/ kg separately and used to conduct the roll towel method in order to assess the effect of antagonists present in the seeds surface on the growth of the sesame seedlings.

The results of this experiment clearly revealed that seeds treated with *Pseudomonas* spp. @ 20 ml per kg of seed enhances the shoot and root length of the ten days old seedlings by recording 12.86 cm and 9.92 cm respectively. Whereas the sesame seeds treated with lower dosage level of 10 ml and high dosage level of 25 ml/kg of seed recorded less root and shoot length of the seedlings (Table 4).

The seeds treated with *B. amyloliquefaciens* @ 20 ml / kg of seed enhances the shoot and root length of the seedlings by recording 12.42 cm and 9.34 cm respectively. Whereas the sesame seeds treated with lower dosage level of 10 ml /kg of seed recorded less root (7.84 cm) and shoot length (10.56 cm) of the seedlings (Table 4). There is no any increase in the root and shoot length of seedlings with proportion to increase in the dosage of liquid formulation used for treating 1 kg of sesame seeds.

B. amyloliquefaciens is a promising candidate with excellent characteristics which have been used for the management of wide range of plant diseases and nematodes (Yap Chin Ann, 2013) [1]. This might be due to their ability to induce growth, defense mechanism in host plant and the production of antibiotic peptides having antifungal and anti-nematode activities (Meena *et al*., 2017) [11]. Application of talc based bio-formulation met with several problems which include their shorter shelf life coupled with lower field performance. In this aspect, liquid formulation had a significant role in maintaining the bacterial population for a longer period of time (maximum of one year) with enhanced protection of the bacteria against adverse environmental conditions.

The nutrient broth amended with glycerol maintained the bacterial population load to a maximum extent of one year. Enhanced survival of the bacterial cells in the liquid formulation might be due to the addition of different chemical amendments to it. Since glycerol holds high amount of water, it protect the cells from desiccation. Manikandan *et al*. (2010) [9] observed the maximum viability of *P. fluorescens* Pf 1 cells in glycerol amended NB. Trehalose is also capable of enhancing the cell tolerance to desiccation by stabilizing the cell membranes (Meena *et al*. 2015) [10].

Different age old cultures of Bbv 57 in liquid formulation had a significant impact in the reduction of fungal colonies when compared with its control. Antibiotics (Levy *et al.*, 1992) [8], siderophores (De Meyer and Hofte, 1997) [4] and certain biotic and abiotic substances (Kasim *et al*., 2013) [6] produced by the PGPR has been regarded as the mechanisms involved in the suppression of plant pathogens. Manikandan *et al*. (2010) [9] proved the mycoparasitic potential of liquid formulation of *P. fluorescens* (Pf1) against *A. solani* and *Fusarium oxysporum* f. sp. *lycopersici in vitro*. Liquid formulation of Bbv 57 isolate tested against nematode - fungal disease complex in

tomato under glass house and field conditions had a significant impact in the reduction of the nematode and fungus population. *B. subtilis* has been reported to be a promising candidate with proven characteristics in the management of nematode fungal disease complex (Meena *et al*., 2015) [10]. Present study proved the efficacy of liquid formulation in maintaining the viability of the bacterial cells for a longer duration of time and also increasing shoot and root length of the seedlings.

Table 1: Population of *B. amyloliquefaciens* in the two days old liquid formulation

S. No.	Population of *B. amyloliquefaciens* (cfu / 0.1ml)			
	24 hrs after plating		48 hrs after plating	
	Dilution level	Population *	Dilution level	Population*
1	10^{-3}	128.33	10^{-3}	254.67
2	10^{-4}	101.67	10^{-4}	196.67
3	10^{-5}	93.33	10^{-5}	184.00
4	10^{-6}	74.33	10^{-6}	146.367
5	10^{-7}	63.33	10^{-7}	128.67
6	10^{-8}	53.00	10^{-8}	107.76
7	10^{-9}	47.33	10^{-9}	92.00
8	10^{-10}	42.67	10^{-10}	83.67
9	Control (plain broth – 10^{-3})	00.00	Control (plain broth – 10^{-3})	00.00

* Mean of three replications

Table 2: Population of *B. amyloliquefaciens* in the seed washes sesame suspension seventy five days after seed treatment

S. No.	Dilution level	Population of *B. amyloliquefaciens* (cfu/0.5 ml) 24 hrs after plating				Population of *B. amyloliquefaciens* (cfu/0.5 ml) 48 hrs after plating			
		10 ml/ kg seed	75 ml/ Kg seed	100 ml/ kg seed	120ml / kg seed	10 ml/ kg seed	75 ml/ Kg seed	100 ml/ kg seed	120ml/ kg seed
1	10^{-3}	UC**	UC	UC	121.33	UC**	UC	UC	126.67
2	10^{-4}	369.33	234.64	123.67	85.66	UC	242.67	126.67	90.67
3	10^{-5}	184.00	106.67	57.00	30.67	347.00	115.67	64.00	36.33
4	10^{-6}	81.33	56.33	41.00	18.00	145.67	60.00	50.67	22.00
5	10^{-7}	14.33	10.00	15.33	8.33	20.33	13.00	9.67	10.33
6	10^{-8}	8.33	5.33	3.33	1.00	10.33	6.67	3.33	9.00
7	10^{-9}	2.33	0.00	0.00	0.00	2.67	0.00	0.00	0.00
8	Control (10^{-3})	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CD (P=0.05)		18.12	15.24	9.98	6.32	18.46	15.78	10.08	6.78

*mean of three replications ** Uncountable number of bacterial colonies

Table 3: Effect of dosage of liquid formulation (LF) of *Bacillus amyloliquefaciens* seventy five days after seed treatment on the growth of the sesame seedlings by roll towel method under laboratory condition

S. No.	Dosage level (LF)	Ten days old seedlings	
		Root length (cm) *	Shoot length (cm) *
1	10ml/kg seed	8.96	11.76
2	75ml/kg seed	8.81	11.99
3	100ml/kg seed	7.98	11.48
4	120ml/kg seed	6.30	9.68
5	Control (10 ml plain broth /kg seed)	5.20	6.33
CD (P=0.05)		0.28	0.32

*Mean of four replications

Table 4: Effect of dosage of liquid formulation of *Pseudomonas spp.* And *B. amyloliquefaciens* ninety days after seed treatment on the growth of sesame seedlings by roll towel method under laboratory conditions

S. No.	Treatments	Ten days old seedlings*	
		Shoot length (cm)	Root length (cm)
1	ST with LF of *Pseudomonas spp.* @ 10 ml/kg seed	10.64	8.18
2	ST with LF of *Pseudomonas spp.* @ 15 ml/kg seed	11.10	8.62
3	ST with LF of *Pseudomonas spp.* @ 20 ml/kg seed	12.86	9.92
4	ST with LF of *Pseudomonas spp.* @ 25 ml/kg seed	12.24	9.44
5	ST with LF of *B. amyloliquefaciens* @ 10 ml/kg seed	10.56	7.84
6	ST with LF of *B. amyloliquefaciens* @ 15 ml/kg seed	11.38	8.32
7	ST with LF of *B. amyloliquefaciens* @ 20 ml/kg seed	12.42	9.34
8	ST with LF of *B. amyloliquefaciens* @ 25 ml/kg seed	12.12	8.88
9	ST with carbendazim @ 2 g/kg seed	11.76	9.12
10	Control (ST with plain broth @ 25 ml/kg seed)	6.62	5.80
CD (P=0.05)		0.68	0.49

* Mean of three replications ST- Seed treatments LF- Liquid formulation

The result of present study agreed with work of Ardakani *et al.*, (2011) [2] reported that *P. florescens* Q18 (B1) and CKK (B2) isolated from cotton soil were used to prepare totally 8 formulations. The green house experimental study result indicated that these antagonists increased the seedling height, root length, seedling dry weight and root dry weight more effectively than control. Talc based bio-formulation of the endophytic *Bacillus strains* EPCO 102 and EPCO 16 and *P. fluorescens* strain Pf1 were tested under greenhouse condition. The bio-formulation was applied through 3 ways *viz.*, seed, soil and foliar spray. Mixed formulation of endophytic bacteria with addition of chitin was found to reduce bacterial blight of cotton. Bacterial strains were also induces the higher levels of chitinase, polyphenol oxidase, peroxidase, phenylalanine ammonia lyase and phenol in cotton plants and reduced the bacterial blight disease.

Pearl millet seeds were treated with talc formulation of *P. fluorescens* against *Sclerospora graminicola* causing downy mildew of pearl millet. The results concluded that, all the test isolates reduced the incidence of downy mildew and growth of the plant was increased both in green house and field condition. In addition, the antagonist enhanced the germination of seeds, seedling vigour, leaf area, height of the plant, capacity of tillering and yield (Kumar and Manga, 2011) [7]. *Streptomyces corchorusii* strain UCR3-16 was isolated from rice soil. This bacterium had antifungal activities against six major rice fungal pathogens and it also produced fungal cell wall degrading enzymes *viz.*, lipase and protease enzymes. In pot culture experiment UCR3-16-treated rice plants showed increased growth and yield. In field experiment also the rice plants were showed enhancement of root and shoot length and grain yield (Tamreihao *et al.*, 2016) [17].

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