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LIQUID FORMULATION OF ENDOPHYTIC *BACILLUS* AND ITS STANDARDIZATION FOR THE MANAGEMENT OF *FUSARIUM* WILT IN TOMATO

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Abstract

Liquid formulation of *Bacillus subtilis* strain EPCO16 was developed to enhance the shelf life and efficacy of the biocontrol agent besides easing the delivery of bioinoculants through microirrigation techniques. Super optimal broth with catabolic repressor (SOC) was found to be effective in promoting the population of *B. subtilis* EPCO16. The greater population level of *B. subtilis* EPCO16 was maintained in SOC amended with glycerol even after 12 months of storage without loss in cell feasibility, antagonistic activity (HCN and siderophore production) and plant growth promotion. The greater efficacy of liquid formulation of *B. subtilis* EPCO16 against *Fusarium* wilt enhanced the fruit yield compared to talc based formulation. The current study offered the successful technology for the development of liquid based formulation for bacterial biocontrol agent.

Introduction

Endophytes are mainly known as beneficial microbes residing inside the plants without harming the host plants. It has several properties such as biocontrol through production of antifungal metabolites and induction of disease resistance and plant growth promotion through fixing biological nitrogen and increasing mineral uptake in plants. Further, the application of endophytes offers an alternate to the use of chemical fungicides in the management of plant diseases. In this regard, our research group has isolated hundreds of endophytes from various agricultural and horticultural plants and tested for their efficacy against wide range of pathogens. Of these, *B. subtilis* EPCO16 has lipopeptide genes *viz., ItuC* gene, *ItuD* gene (Iturin); *BmyA* gene (Bacillomycin A), *BacD* gene (Bacillomycin D), *BacAB* gene (Bacilysin) and *FenD* gene (Fengycin). The presence of lipopeptide antibiotics in *B. subtilis* EPCO16 inhibited the mycelial growth (46.04%) of *F. oxysporum* f. sp. *lycopersici* (Fol) under *in vitro* (Ramyabharathi and Raguchander 2014). The talc based powder formulations are popular in India and elsewhere, the shorter shelf-life and reduced efficacy during longer storage periods (Singh *et al.* 2006) necessities the development of alternate formulation having longer shelf life. Further, the application of talc based bioformulations through microirrigation techniques encountered the problems such as blockage of nozzles and uneven distribution of bio-inoculants. In these circumstances, the development of liquid formulation comes in handy as it facilitates the abundant production of spores at a shorter period (Harman *et al.* 1991). In addition, it has been demonstrated that the development of liquid formulation has several advantages including high cell count, zero contamination, longer shelf life, greater protection against environmental stresses and increased field efficacy (Hegde 2002, Vendan and Thangaraju 2006). In liquid formulation, the microbial organisms are present in a dormant form and after application in the field, the dormant form gives

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rise to active cells. This helps to increase the shelf life of liquid bioformulation for more than one year (Vendan and Thangaraju 2006). Thus, the current study was carried out (i) to find out the suitable liquid media for the growth and maintenance of *B. subtilis* EPCO16 population at different times of storage, (ii) to evaluate the properties of liquid formulation *B. subtilis* EPCO16 at different storage intervals and (iii) to standardize the dose and application methods for the management of *Fusarium* wilt in tomato as a model crop.

Materials and Methods

The endophytic biocontrol strain *Bacillus subtilis* EPCO16 (Accession Number. EF139864) was obtained from the Culture Collection Centre, Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.

B. subtilis EPCO16 was grown in SOC broth, after 72 hrs the population load was 1×10^{11} cfu/ml. The cells were harvested by centrifugation at 5000 rpm at 4°C and washed three times with 100 mM potassium phosphate buffer solution. 25 ml of this culture was inoculated into 1 l of SOC liquid medium in combination with different chemical amendments to increase the survival of *B. subtilis* cells. The chemical amendments *viz*., trehalose at 10 mM, polyvinylpyrollidone (PVP) at 2% and glycerol at 10 mM were added to 1 l of SOC broth separately as per the method described by Vendan and Thangaraju 2006. An uninoculated control was maintained for each broth and the flasks were incubated at room temperature. The broth culture was analyzed for viable cell population at monthly intervals up to 365 days.

A nine mm mycelial disc of the tomato wilt pathogen *Fusarium oxysporum* f. sp. *lycopersici* (Fol) was placed in the centre of the Petri plate. Sterile Whatman No. 40 filter paper discs with six mm dia were placed one cm away from the edge at four sides centering around the fungal disc. Twenty five µl of broth cultures of different days stored *B. subtilis* EPCO16 were dropped over the filter paper discs. Observations were taken after five days for the presence of inhibition zone over the pathogen. Control was maintained with the sterile distilled water instead of bacterial inoculum.

The plant growth-promoting activity of different days stored *B. subtilis* was assessed based on the seedling vigour index by the standard roll towel method (ISTA 1993).

Production of siderophore by different days stored cultures of *B. subtilis* EPCO16 was tested by plate assay method as described by Schwyn and Neilands (1987). Hydrogen cyanide production was estimated according to Miller and Higgins (1970). The results were calculated up to 365 days.

To standardize the dosage of liquid formulation of EPCO16 for seed treatment and to assess the survival on different formulations of EPCO16 in talc and liquid, the following method was adopted with varied inoculum levels as described by Vendan and Thangaraju (2006). The seeds of tomato cv. PKM1 were taken for this study. The surface sterilized seeds were coated with the inoculum at three levels *viz.*, 5, 10 and 15 ml (containing population load of 10^{10}/ml) per kg of seed. An equal volume of sterile water or rice gruel was added with the inoculum and inoculated directly to the seed. The talc based inoculums was applied at 10 g (1 g containing the population load of 6.5×10^8) per kg of seed. Inoculated seeds were aseptically kept at room temperature.

Immediately after the seed inoculation with liquid or talc formulations, sample of 10 seeds were assayed to determine the number of viable bacterial cells on the seeds. Each seed sample was placed into a 25 ml flask containing 10 ml diluents (0.85% NaCl and 0.01% Tween 80), vortexed for 5 min. Samples were serially diluted and 100 µl of 10^{-6} – 10^{-9} dilutions were spread plated on NA agar plates. The remaining seeds were placed in Petri dish at room temperature (28 - 30°C).

Ten seed samples were assayed for EPCO16 population as above mentioned at 6, 12, 24, 36 and 48 hrs after inoculation and the results were expressed as colony forming units (cfu) per seed.

To standardize the dosage of liquid formulation of *Bacillus* for seedling dip and to assess its survival on seedlings following method was adopted with varied inoculum levels. The tomato seedlings (PKM1) were raised in sterile soil and the roots were washed with sterile water. The liquid formulations were inoculated at three levels *viz*., 250, 500 and 750 ml/ha seedlings with 25 litre of sterile water. Talc based formulation at 2.5 kg was also mixed with 25 litre of sterile water to make a slurry and the seedlings required for one ha was dipped in the slurry and kept in shade. After 30 min of seedling dip, 10 seedlings were taken from a treatment and the root portions were cut and mixed thoroughly. From that 1 g of root portion, *Bacillus* cells were serially diluted and plated on NA agar plates. The survival of EPCO16 cells at 0, 1, 2, 3 and 6 hrs intervals was assessed and the results were expressed as cfu/g of root.

Glasshouse studies were conducted to test the efficacy of talc and liquid based bioformulations containing EPCO16 against *F. oxysporum* f. sp. *lycopersici.* The talc and liquid formulation of *B. subtilis* EPCO16 was tested for their efficacy on growth promotion. The yield of ripened fruits per plant and per cent disease incidence was also observed.

The field experiment was conducted in tomato growing region *viz.,* Devarayapuram, Coimbatore, Tamil Nadu, India to test the efficacy of *B. subtilis* liquid formulation and *Bacillus* talc based bio-formulation against *Fusarium* wilt disease. The trial was laid out with randomized block design with three replications. Production practices were followed as per the recommendation of crop protection guide of Tamil Nadu Agricultural University, Coimbatore. The foliar application was done at 15 days interval (four sprays per season). In the field, the talc product at 2.5 kg/ha was mixed with the small quantity of water and it was delivered to the field with the help of drip irrigation system along with the water covering one ha of land at 30 days after planting. Like that liquid product at 1000 ml/ha was delivered to the field through drip irrigation system. Each treatment consisted of three replications and each replication approximately consisted of 100 plants. The disease incidence was assessed based on the number of plants infected relative to the total number of plants observed in a randomized manner. After harvesting the crop, fruit yield was recorded.

The data were statistically analyzed using the IRRISTAT version 92 developed by the International Rice Research Institute Biometrics unit, the Philippines (Gomez and Gomez 1984). The percentage values of the disease index were arcsine transformed. Data were subjected to ANOVA and means were compared by DMRT.

Results and Discussion

Among the chemical amendments, addition of glycerol to SOC broth (10 mM) recorded higher level of sporulation throughout the period of observation followed by trehalose (10 mM) (Table 1). Similarly in glycerol amended phosphate buffer medium the population of *B. subtilis* EPCO16 remained higher up to 365 days of incubation. Hence, addition of chemical amendments *viz.,* glycerol and trehalose in SOC broth found to be best in supporting the population survival of EPCO16.

The stored EPCO16 culture from 2nd day to 365 days inhibited the growth of *F. oxysporum* f. sp. *lycopersici* up to 44.44% (Table 2). Regarding growth promotion the two days old culture of EPCO16 recorded higher germination of 92%, increased shoot and root length of 6.1 and 13.77 cm respectively with vigour index of 1833.83 in compare to talc formulation of EPCO16 which recorded vigour index of 1713.07. The growth promoting activity was slightly decreased towards 365 days but higher than control (Table 2).

The intensity of the siderophore production by EPCO16 was higher up to 120 days and there after the intensity started decreasing slightly from 150 days onwards up to 365 days. For HCN results showed that the culture up to 180 days changed the yellow color of the filter paper to dark brown indicating HCN production. The culture stored above 180 to 365 days did not show the production of HCN (Table 2).

Table 1. Comparative study on suitability of chemical amendments in SOC for *B. subtilis* EPCO16.

Days	EPCO population (CFU/ml)*			
	Glycerol	Trehalose	PVP	Broth alone
1st	4.5×10^{10}	4.4×10^{10}	4.3×10^{10}	4.2×10^{10}
5th	3.0×10^{12}	3.0×10^{12}	2.9×10^{12}	2.3×10^{12}
15th	1.2×10^{12}	1.1×10^{12}	4.3×10^{11}	4.0×10^{11}
45th	4.2×10^{11}	1.1×10^{11}	6.5×10^{10}	3.1×10^{10}
60th	8.2×10^{10}	4.3×10^{10}	2.2×10^{10}	1.0×10^{10}
90th	7.2×10^{10}	3.3×10^{9}	1.2×10^{10}	1.0×10^{9}
120th	5.6×10^{10}	2.3×10^{9}	2.2×10^{9}	2.3×10^{8}
150th	3.9×10^{10}	1.3×10^{10}	1.3×10^{10}	2.0×10^{8}
180th	3.2×10^{10}	1.2×10^{10}	1.2×10^{9}	2.0×10^{7}
240th	2.5×10^{10}	1.4×10^{9}	1.3×10^{8}	1.0×10^{7}
365th	2.4×10^{10}	1.3×10^{9}	1.2×10^{8}	1.0×10^{7}

*****Values are mean of four replications.

Table 2. Growth promotion and antagonistic activity of different day old liquid formulation of *B. subtilis* EPCO16.

Days old liquid formulation	FoI		*Vigour index	Siderophore	HCN
	Mycelial growth of the pathogen (mm)	Per cent inhibition over control			
2nd day	47.20^{a}	47.55^{a}	1833.83^{a}	+ + +	+ +
7th "	47.20^{a}	47.55^{a}	1665.16^{bc}	+ + +	+ +
15th "	48.00^{a}	46.66^{ab}	1628.92^{bcd}	+ + +	+ +
30th "	48.00^{a}	45.88^{ab}	1584.19^{b-e}	+ + +	+ +
45th "	48.00^{a}	46.66^{ab}	1578.85^{b-e}	+ + +	+
60th "	48.00^{a}	46.66^{ab}	1554.83^{cde}	+ + +	+
90th "	48.00^{a}	46.66^{ab}	1572.63^{cde}	+ + +	+
120th "	48.00^{a}	46.66^{ab}	1539.86^{def}	+ + +	+
150th "	48.00^{a}	46.66^{ab}	1500.14^{efg}	+ +	+
180th "	48.00^{a}	46.66^{ab}	1428.192^{g}	+ +	+
240th "	49.00^{b}	45.55^{ab}	1416.96^{g}	+ +	-
365th "	50.00^{c}	44.44^{b}	1396.40^{h}	++	-
Talc formulation (110th day)	-	-	-	-	-
Control (Sterile water)	90.00^{d}	0.0^{c}	964.96^{i}	-	-

+ + +, strong production; ++, medium production; +, low production; -, no production. Values are mean of three replications; Means followed by a common letter are not significantly different at 1% level by DMRT.

The survival and adherence of *B. subtilis* EPCO16 in talc and liquid formulations at varied inoculum levels on tomato seeds showed that the population of EPCO16 adhered to the seeds was gradually reduced from 0 to 48 hrs of incubation in both the formulation. Liquid formulation of

B. subtilis EPCO16 mixed with rice gruel performed better than the carrier based one. Inoculum level of 15 ml/kg of seeds has recorded maximum population when compared to 10 and 5ml/kg of seeds (Table 3). The liquid formulation + sterile water at 500 ml/ha recorded maximum adherence and extended survival of EPCO16 on the roots of tomato seedlings followed by the talc formulation. The initial population of EPCO16 was 2.2×10^{11} cfu/g of root which was gradually decreased to 2.2×10^{5} cfu/g of root with the increase of incubation period up to 6 hrs (Table 4).

Table 3. Colonization ability of liquid formulation of *B. subtilis* EPCO16 in tomato seeds with different adhesives.

Treatments	Population of *Bacillus subtilis* (cfu/seed)					
	0 hr	6 hrs	12 hrs	24 hrs	36 hrs	48 hrs
LF of EPCO16 + sterile water at 5 ml/kg seed	4.2×10^{10}	4.5×10^{9}	3.7×10^{8}	3.9×10^{6}	2.4×10^{3}	0.0
LF of EPCO16 + rice gruel at 5 ml/kg seed	4.4×10^{10}	5.0×10^{9}	4.2×10^{8}	4.0×10^{6}	2.4×10^{3}	0.0
LF of EPCO16 + sterile water at 10 ml/kg seed	3.0×10^{11}	2.2×10^{11}	4.8×10^{8}	3.1×10^{7}	2.1×10^{3}	2.0×10^{2}
LF of EPCO16 + rice gruel at 10 ml/kg seed	3.2×10^{11}	2.7×10^{11}	5.0×10^{8}	4.1×10^{7}	3.1×10^{3}	3.1×10^{2}
LF of EPCO16 + sterile water at 15 ml/kg seed	3.2×10^{11}	2.4×10^{11}	4.9×10^{8}	3.2×10^{7}	3.2×10^{2}	2.2×10^{2}
LF of EPCO16 + rice gruel at 15 ml/kg seed	3.8×10^{11}	2.8×10^{11}	6.1×10^{8}	4.2×10^{7}	4.1×10^{3}	3.4×10^{2}
TF of EPCO16 at 10 g/kg of seed	2.4×10^{10}	2.1×10^{9}	2.9×10^{8}	2.4×10^{6}	1.0×10^{2}	0.0

LF - Liquid formulation; TF - Talc formulation.

Table 4. Root colonization by liquid formulation of EPCO16 on tomato seedlings after seedling dip method.

Treatments	Population of *Bacillus subtilis* (cfu/g of root on dry weight)				
	0 hr	1 hr	2 hrs	3 hrs	6 hrs
LF of EPCO16 at 250 ml/ha	2.3×10^{11}	2.2×10^{11}	2.0×10^{9}	3.0×10^{7}	2.7×10^{5}
LF of EPCO16 at 500 "	2.2×10^{11}	2.9×10^{11}	3.1×10^{8}	2.7×10^{8}	2.2×10^{5}
LF of EPCO16 at 750 "	2.1×10^{11}	2.5×10^{10}	2.2×10^{9}	2.6×10^{8}	2.5×10^{5}
TF of EPCO16 at 2.5 kg/ha	2.2×10^{10}	2.9×10^{10}	2.0×10^{8}	2.4×10^{7}	2.0×10^{5}

LF - Liquid formulation; TF - Talc formulation.

Combined application of seedling dip + soil application + foliar spray with liquid formulation recorded the lowest disease incidence of 17.46% with maximum plant height of 87.60 cm, fruit yield of 280.46 g/plant after 60 days after transplanting (Table 5) compare to untreated control.

Combined application of seedling dip (500 ml/ha) + soil application (1000 ml/ha) + foliar spray (1000 ml/ha) of EPCO16 liquid formulation decreased the *Fusarium* wilt up to 68.42% with the yield of 36.67 t/ha this was followed by combined application of mancozeb which recorded 63.16% disease reduction over the control. In control plot the *Fusarium* wilt incidence was highest with 29.75% (Table 5).

The importance of liquid formulated biocontrol agents for disease management has already been reported in Horticultural crops both in glasshouse and field condition (Sriram *et al.* 2011). In order to survive in the commercial market and to achieve the desired effect of beneficial

organisms, the strains in microbial products must be cost-effectively formulated to remain dormant, to make them to survive under high and low temperatures of the environment during transportation and storage. Compare to *Pseudomonas*, the endospores produced by *Bacillus* species make them to survive without nutrients. In this study, addition of glycerol to SOC medium preserved the viability of EPCO16 cells in liquid formulation for the storage period of 12 months. This finding is similar with that of Gervasio *et al.* 2009 who reported the use of glycerol as a carbon source to obtain valuable microbial products. Hence, the addition of glycerol helps in maintaining higher moisture content in the formulation and protected the viable propagules from the reduced water activity during shelf-life. In the bio-efficacy studies, even after 12 months of storage, the liquid formulated *B. subtilis* EPCO16 showed significant reduction in the mycelial growth of *F. oxysporum* f. sp. *lycopersici* under *in vitro*. Similarly for growth promotion the vigor index of tomato seedlings was higher in seeds treated with 365 days stored liquid formulated biocontrol agent compared to untreated control.

Table 5. Efficacy of liquid bioformulation of *B. subtilis* EPCO16 against *Fusarium* wilt of tomato cv. PKM1.

Treatments	Glasshouse trial				Field trial			
	Plant height (cm)	Root length (cm)	Fruit yield g/plant	Per cent incidence	*Plant height (cm)	*Root length (cm)	*Yield (t/ha)	Per cent incidence
Seed treatment of LF at 10 ml/kg	67.10^{d}	32.26^{j}	181.96^{de}	28.20^{ef} (32.63)	72.02^{cd}	35.01^{f}	30.89^{f}	4.61^{d} (12.39)
Seed treatment + seedling dip of LF at 500 ml/ha	77.00^{b}	37.43^{c}	246.90^{b}	18.98^{b} (26.24)	77.20^{b}	37.34^{d}	32.50^{d}	4.01^{c} (11.55)
Seed treatment + seedling dip + soil application of LF at 1000 ml/ha	87.60^{a}	42.69^{a}	280.46^{a}	17.46^{a} (25.22)	87.61^{a}	42.80^{a}	36.67^{a}	3.00^{a} (9.97)
Seed treatment of TF at 10 g/kg	73.26^{bc}	35.65^{f}	182.53^{de}	27.96^{ef} (32.06)	72.56^{bcd}	35.28^{e}	29.33^{g}	5.01^{e} (12.94)
Seed treatment + seedling dip of TF	74.23^{bc}	36.21^{e}	193.06^{d}	20.96^{c} (27.68)	74.54^{bc}	36.67^{e}	31.67^{e}	5.76^{g} (13.89)
Seed treatment + seedling dip + soil application of TF	83.66^{a}	40.62^{b}	273.23^{a}	18.23^{a} (25.41)	83.67^{a}	40.96^{c}	33.20^{c}	3.67^{b} (11.04)
Seed treatment with mancozeb at 0.2%	70.00^{cd}	34.34^{h}	171.73^{e}	28.46^{ef} (32.79)	70.10^{cd}	34.05^{g}	28.33^{h}	5.33^{f} (13.34)
Seed treatment + seedling dip of mancozeb	74.23^{bc}	36.43^{d}	230.26^{c}	27.10^{e} (31.54)	69.13^{d}	33.45^{h}	27.33^{i}	4.00^{c} (11.53)
Seed treatment + seedling dip + soil application of mancozeb	70.00^{cd}	33.98^{i}	232.16^{c}	24.40^{d} (29.11)	87.62^{a}	42.39^{b}	35.00^{b}	3.50^{b} (10.78)
Control	62.30^{e}	30.52^{k}	188.96^{d}	59.50^{g} (50.13)	57.59^{e}	28.79^{i}	25.67^{j}	29.75^{h} (33.05)

LF - Liquid formulation of EPCO16; TF - Talc formulation of EPCO16. Values are the mean of three replications; In a column, means followed by a common letter are not significantly different at the 5% level by DMRT; Values in the parentheses are arscine transformed values.

Competition for iron by siderophore production has long been recognized as an important antagonistic trait found in many of the bacterial biocontrol agents against plant pathogens (Xianmei *et al.* 2011). The production of iron chelating agents and HCN by EPCO16 cultures in

the liquid formulation more than 180 days of storage has been reported. This determined the *B. subtilis* EPCO16 effectiveness in liquid-based formulation. The application of liquid formulated EPCO16 at rate of 10 ml/kg of seeds with equal volume of rice gruel and 500 ml/ha with sterile water as seedling dip recorded the maximum viability of EPCO16 cells on seeds and seedling roots, respectively. Similarly Gomathy *et al*. (2007) reported the germination of *Bacillus* spores was higher in nutrient broth followed by rice gruel and sterile lignite as it had nutrients, which helped the bacterial spores to germinate. The application of liquid formulation with rice gruel favoured adherence of greater numbers of cells on the seeds, and was attributed to its sticky nature and nutrient contents (Kundu and Gaur 1981). Thus, liquid inoculants can support cell survival on seed better than the solid carrier formulation (Vendan and Thangaraju 2006). Further, the efficacy of many antagonists against plant pathogens and on plant growth promotion is directly related to the number of antagonist cells inoculated (Hofstein *et al.* 1994).

Further, the efficacy of liquid formulation EPCO16 against *Fusarium* wilt incidence under glasshouse and field conditions has been reported very well in this study. Considering the benefit of extended shelf life and marketability of the product, the commercial units producing *Bacillus* liquid formulations can use this simple procedure of adding glycerol and get extended shelf-life for their products. This will ensure good quality and viable products in the market available for the farmers who are the end users. *B. subtilis* when used as biocontrol agent not only will reduce disease incidence but will help in getting good plant growth promotion thereby indirectly helping in increased yield parameters (Ramyabharathi *et al.* 2012). Further, the lipopeptides, namely the family of surfactin, iturin and fengycin produced by *Bacillus* species offer a wide range of antibiotic potential against phytopathogens, including bacteria, fungi and oomycetes. Recent investigations have shown that these lipopeptides can also influence the ecological fitness of the producing strain in terms of root colonization (thereby persistence in the rhizosphere) and also have a key role in the beneficial interaction of *Bacillus* species with plants by stimulating host defence mechanisms (Ongena and Jacques 2008).

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