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EMPLOYING FORMULATIONS OF BENEFICIAL RHIZOBACTERIA TO IMPROVE GROWTH AND HEALTH OF TOMATO (*LYCOPERSICON ESCULENTUM* MILL.)

GOWTHAM H. G.[1], HARIPRASAD P.[2] AND NIRANJANA S. R.[1]*

[1]Department of Studies in Biotechnology, University of Mysore, Manasagangotri, Mysore – 570006, Karnataka, INDIA.
[2]Centre for Rural Development and Technology, Indian Institute of Technology Delhi, Hauz Khas, New Delhi – 11001, INDIA.

ABSTRACT

In the present study, we attempted to improve the growth and health of tomato (*Lycopersicon esculentum* Mill.) using talc based formulations of two beneficial rhizobacteria (*Bacillus subtilis* PSIRB2 and *Pseudomonas aeruginosa* 2apa). These isolates were well characterized for their beneficial traits in our previous studies. Shelf life of talc-formulation of isolate 2apa was found stable up to 60 days, afterwards a drastic decrease was observed with the storage period at room temperature. The population of PSIRB2 decreased gradually throughout the experimental period of 120 days to 0.25×10^7 cfu/g formulation. Growth promotion studies under laboratory and greenhouse conditions revealed that the talc-formulations of both the test bacteria as good as fresh cultures in increasing the growth variables, in comparison with control. Similarly, PSIRB treated seedlings (both talc-formulation and fresh culture) recorded significantly ($P \leq 0.05$) higher nutrient uptake. Under greenhouse conditions isolate 2apa recorded significant ($P \leq 0.05$) disease protection in both talc-formulation and fresh culture form against Fusarium wilt and Early blight diseases of tomato.

KEY WORDS: *Talc-formulation, Tomato, Rhizobacteria, Shelf-life.*

NIRANJANA S. R. *

Department of Studies in Biotechnology, University of Mysore, Manasagangotri, Mysore – 570006 Karnataka, INDIA.

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INTRODUCTION

Even though agricultural production has increased in the past decades, but pressure is still on to cope up with the increasing population across the world. Farmers' are becoming more and more dependent on agrochemicals, as a reliable and cheap method for enhancing crop productivity and managing plant diseases. On the other hand increase the use of agrochemicals causes several negative effects, *i.e*., soil degradation, eutrophication, development of resistance in agricultural pests, etc. There is now overwhelming evidence that some of these chemicals do pose potential risk to humans and other life forms and unwanted side effects to the environment [1,2,3]. The world-wide death and chronic illness due to pesticide poisoning is about 1 million per year [4]. The Endosulfan tragedy at Kasaragod, Kerala is the best example of impact of pesticides on humans and the environment. In the existing scenario an ecofriendly and sustainable alternatives to chemical fertilizers and pesticides are much needed. Nature harbors both harmful and beneficial microbes which affect the plant growth and health both negatively and positively. Use of excess agrochemicals not only affects the harmful microbes, with the time it also reduced the beneficial microbes in the soil. In order to restore the soil fertility, it is important to introduce some of the microbes back to soil. Hence, the use of beneficial micro-organisms as bio-fertilizers and biocontrol agents has become more important in recent years in order to improve plant growth and manage plant diseases but also to avoid environmental pollution [5]. Plant growth promoting rhizobacteria (PGPR) are known to promote plant growth and suppress plant diseases by various mechanisms [6]. The major factor that goes into success of biocontrol programme is the effectiveness with which the biocontrol agents are delivered to the environment. Bio-pesticides and bio-fertilizers formulations usually prepared as carrier-based inoculants containing effective micro-organisms. Incorporation of micro-organisms in carrier material enables easy-handling, long-term storage and high effectiveness of bio-pesticides and bio-fertilizers. Among various types of bio-pesticides and bio-fertilizers, bacterial inoculant is one of major groups which includes nitrogen-fixing rhizobacteria, phosphate-solubilizing bacteria, potash mobilizing bacteria and so on. In most of the studies, prepared bacterial inocula either liquid suspensions [7,8,9] or bacteria mixed with peat [10,11,12] have been applied. Kloepper and Schroth [13] demonstrated the potentiality of talc to be used as a carrier for formulating rhizobacteria. Further, different carrier materials such as Peat, Kaolinite, Lignite, Vermiculite and Stickers were used by Vidyasekaran and Muthamilan [14] to develop a suitable formulation of fluorescent Pseudomonads. The purpose of the present study was to develop talc based formulations of well characterized beneficial rhizobacteria and their evaluation under laboratory and greenhouse conditions for their plant growth promoting and fungal disease suppressing ability (Fusarium wilt and Early blight) which can be used as alternative to agro-chemicals in sustainable tomato production.

MATERIALS AND METHODS

Micro-organisms and cultural conditions

Two rhizobacterial isolates (*Bacillus subtilis* PSIRB2 and *Pseudomonas aeruginosa* 2apa) were originally isolated from rhizospheric soil samples of tomato and characterized for the beneficial traits [6,15]. Both the selected isolates were maintained on nutrient agar (NA) slants at 4° C for routine work. For long term storage rhizobacterial isolates were maintained in 40% glycerol at –80° C. The fungal pathogens *Fusarium oxysporum* f. sp. *lycopersici* and *Alternaria solani* were collected from Department culture collection, Department of Biotechnology, University of Mysore, India. Both the pathogens were maintained on potato dextrose agar (PDA) slants throughout the experimental period.

Preparation of Talc based bioformulation

Talcum powder formulation was developed by following modification the method of Vidhyasekaran and Muthamilan [14] using a mixture of 10 g of carboxymethyl cellulose (CMC) and 1 kg of talc. The pH was adjusted to 7 by adding calcium carbonate ($CaCO_3$) and placed in a metal tray followed by thorough mixing. The mixture was autoclaved for 45 min at 121.5° C (15 lb/inch^2) in two consecutive days. Rhizobacterial inoculum was prepared by growing the selected bacteria in nutrient broth for 36 h on rotary shaker (150 rpm) at 32±2° C. The bacteria was pelleted by centrifugation and washed using Phosphate buffer saline (PBS). The bacterial concentration was adjusted using spectrophotometer to 1×10^{16} cfu/ml by using the standard graph of OD_{610} vs plate count (data not shown). Five hundred ml of bacterial suspension containing 1×10^{16} cfu/ml was added to 1 kg of talc material and mixed well under sterile conditions to get an inoculum density of 1×10^8 cfu/g talc powder. Formulation was packed in polythene bags, sealed and stored at room temperature with a moisture content of 35%.

Determining the shelf life of developed formulation

To determine the efficacy of selected bacterial isolates to survive in carrier material under room temperature, 1 g of formulation was drawn at regular time intervals *viz*., 0, 15, 30, 45, 60, 75, 90, 105 and 120 days after storage. Each formulation was serially diluted and suitable dilutions were spread plated onto NA. The plates were incubated for 36 h at 32±2° C and the bacterial colony in each plate was counted and tabulated as cfu/g formulation.

Efficacy of talcum formulation on plant growth of tomato under laboratory and greenhouse conditions

The seeds of tomato (*Lycopersicon esculentum* Mill.) cultivar 'PKM-1' obtained from local seed agencies, Mysore. Seeds were surface-sterilized with 1% sodium hypochlorite for 30 s and then rinsed in sterile distilled water, blot dried and used for the experiments. Surface sterilized tomato seeds were first wetted with water and mixed thoroughly with talc-based formulation (10 g/kg seed) of each bacterial isolates. Seeds were then spread on a blotter sheet, shade-dried and used for the experiments. Seeds treated with talcum powder amended with CMC serve as control. For comparative analysis, fresh culture of bacteria seed treated as per

the procedure given by Hariprasad *et al*.,[6] and used throughout the experiment. Plant growth promotion studies under laboratory was done following between paper method [16] and root length, shoot length and percent of germination was recorded. Further, vigor index was calculated using the formula: VI = (Mean root length + Mean shoot length) × % Germination [17]. In greenhouse, further to analyze the plant growth at early stage, treated and control seedlings were sown into earthen pots containing sterilized potting mixture, soil: FYM: coir pith at the ratio of 2:1:1 (v/v/v). After 10 days, the seedlings were thinned by leaving six seedlings/pot. The seedlings were maintained under greenhouse conditions and watered at regular intervals. Twenty five day old seedlings were removed carefully without damaging the root system, washed under running tap water and blot dried. Root length, shoot length, fresh weight and dry weight were calculated for each treatment. In one more set of experiments, the plants were allowed to grow for 75 days (one plants/pot). Plant height, fresh weight and chlorophyll content for each treatment were determined for 75 day-old-plants [18]. Briefly, 100 mg of fresh leaves were crushed in 20 ml of 80% acetone and the extract centrifuged at 1000 rpm for 10 min. Absorbance of the supernatant was recorded at 645 and 663 nm in a spectrophotometer. Total chlorophyll content was expressed as mg/g of each sample.

$$\text{Total chlorophyll (mg/g tissue)} = [20.2\ (A_{645}) - 8.02\ (A_{663}) \times VW]/\ 1000$$

where A=absorbance at the given wavelength, W = weight of fresh leaf sample, V = final volume chlorophyll solution.

For early plant growth studies, replicates of each treatment contain eight pots with six seedlings/pot and for each treatment such three replicates were maintained. For 75 day old plants, each replicate of the treatment contains six plants and four replicates were maintained for each treatment and the experiment was performed twice.

Soil and plant nutrient analyses

Total N was determined by Kjeldahl method [19]. Plant tissue was digested in a mixture of 15 ml of Perchloric acid ($HClO_4$) and 5 ml of Nitric acid (HNO_3), and phosphorus was determined spectrophotometrically by vando-molybdate method [20]. Other elements were determined by atomic absorption spectrophotometry [21]. All the above said analyses were conducted in Soil Analysis Lab, Gandhi Krishi Vignayan Kendra (GKVK), V.C. Farm, Mandya, Karnataka.

Efficacy of talcum formulation on Fusarium wilt and Early blight incidence in tomato under greenhouse conditions

Seedlings from control and treated seeds were raised as explained above, after transplantation 30 day-old-seedlings were challenge inoculated with conidial suspension (1×10^5 conidia/ml) of *F. oxysporum*. Wilt incidence was recorded up to 60 days after challenge inoculation. Conidial suspension of *A. solani* (5×10^4 conidia/ml) was sprayed onto leaf of 30 day-old-seedlings until runoff for Early blight disease. Appearance of typical leaf spot symptoms was recorded up to 30 days after challenge inoculation. For each experiment four pots per replication were arranged randomly and maintained with eight replications and the experiment was repeated twice.

STATISTICAL ANALYSIS

Data obtained from laboratory and greenhouse were analyzed for significant differences by analysis of variance (ANOVA) with mean separation using the least significant difference (LSD) ($P \leq 0.05$) by Fisher's protected LSD test, employing the statistical tool SPSS version 16.0 (SPSS Inc., Chicago, IL).

RESULTS

Shelf life of talc-formulation

Talc-formulation of selected rhizobacterial isolates were prepared using talc powder as carrier material. Initial rhizobacterial population was 1×10^8 cfu/g formulation as determined by serial dilution method on defined medium. Shelf life of all the selected PGPR formulations was found to be decreasing along with the storage period (Fig. 1). The initial high population of rhizobacteria in the talc formulation was not necessarily sustained during storage. Population of PSIRB2 decreased gradually throughout the experimental period. After the storage of 120 days at room temperature, population of PSIRB2 decreased to 0.25×10^7 cfu/g formulation. Whereas in case of isolate 2apa the stability of population was maintained up to 60 days of storage, after that a drastic decline in the bacterial population was observed and at the end of the experimental period the cfu was 1.7×10^4 /g formulation.

Efficacy of Talc based bioformulations in increasing the plant growth and nutrient content in tomato under greenhouse conditions

Seed treatment with bioformulations of isolates 2apa and PSIRB2 significantly ($P \leq 0.05$) increased the root, shoot length and vigor index of tomato seedlings when compared to untreated control under laboratory conditions (Table 1). Similarly, when 25-day-old seedlings were analyzed under greenhouse conditions, a significant increase in root length, shoot length, fresh weight and dry weight was recorded with both rhizobacterial talc-formulations in comparison with untreated control (Table 1). The efficacy of talc formulations was found to be equivalent to its fresh culture. The accumulation of nutrients in tomato seedlings was tabulated in the Table 2. Isolates PSRIB2 and 2apa in the form of both fresh culture and talc-formulations significantly ($P \leq 0.05$) enhanced the accumulation of N, P and K in shoot dry matter of tomato plants. In case of Ca and Mg, a similar pattern of accumulation was observed. Both fresh cultures and talc-formulations performed similarly in case of nutrient accumulation (Table 2). Among the two talc-formulations tested, PSIRB2 was found effective in enhancing nutrient uptake ability of tomato plants. In 75 day-old-seedlings, increased fresh weight was noticed with treatments of talc-formulations (Table 3). Whereas, the chlorophyll content of tomato seedlings was not affected by talc-formulation treatments (Table 3). The talc-formulation of isolate 2apa significantly ($P \leq 0.05$) controlled both Fusarium wilt and Early blight diseases (12 and 17% respectively) compared to control (76%,

Fusarium wilt and 80%, Early blight). Formulation of isolate PSIRB2 completely failed to offer protection against both the fungal diseases studied under greenhouse conditions. In all cases, performance of both fresh cultures and talc-formulations were not significantly ($P \leq 0.05$) different (Table 3).

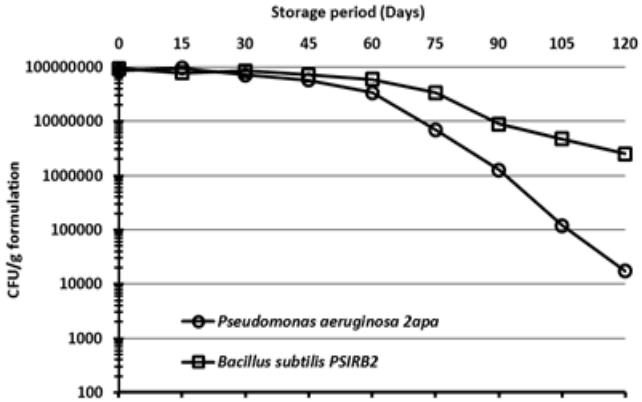

Figure 1
Survival of selected beneficial rhizobacteria in talcum powder formulation at room temperature.

Table 1
Effect of seed treatment with formulation on early plant growth of tomato under laboratory and greenhouse conditions.

Isolates	Treatment	Under laboratory conditions				Under greenhouse conditions			
		MRL (cm)	MSL (cm)	Germination (%)	VI	MRL (cm)	MSL (cm)	FW (g/s)	DW (g/s)
Control		6.6±0.11^{b}	5.8±0.17^{b}	76±2.15^{a}	942±12.1^{b}	13.6±0.34^{c}	11.3±0.17^{b}	0.425±0.04^{b}	0.050±0.005^{b}
2apa	FC	8.0±0.28^{a}	7.1±0.17^{a}	79±2.30^{a}	1193±45.2^{a}	14.0±0.34^{bc}	13.0±0.28^{a}	0.632±0.01^{a}	0.073±0.001^{a}
	FO	8.0±0.23^{a}	8.0±0.28^{a}	78±3.46^{a}	1248±23.5^{a}	15.5±0.17^{ab}	13.5±0.17^{a}	0.601±0.04^{ab}	0.065±0.001^{ab}
PSIRB2	FC	8.2±0.11^{a}	7.7±0.05^{a}	78±1.73^{a}	1240±39.4^{a}	15.9±0.51^{a}	13.6±0.11^{a}	0.574±0.01^{ab}	0.063±0.002^{ab}
	FO	8.1±0.05^{a}	7.9±0.23^{a}	78±3.46^{a}	1248±13.8^{a}	13.9±0.57^{bc}	13.2±0.05^{a}	0.479±0.08^{ab}	0.053±0.007^{b}

FC: fresh culture; FO: formulation, MRL: Mean root length, MSL: Mean shoot length, VI: Vigor index, FW: Fresh weight, DW: Dry weight. Values are the mean with in the column sharing the same letters are not significantly different according to Tukey's HSD at $P \leq 0.05$.

Table 2
Effect of seed treatment with formulations of selected PGPR on nutrient accumulation in tomato plants under greenhouse conditions.

Isolates	Treatment	Dry matter (mg/g)				
		N	P	K	Mg	Ca
Control		12.4±0.23^{c}	1.9±0.23^{b}	20.1±1.15^{b}	4.2±0.11^{b}	20.4±1.50^{a}
2apa	FC	21.1±2.07^{a}	2.0±0.17^{b}	23.8±0.98^{ab}	4.8±0.18^{ab}	24.5±2.59^{a}
	FO	18.0±1.50^{ab}	2.0±0.11^{b}	24.0±0.98^{ab}	4.6±0.14^{ab}	22.8±2.25^{a}
PSIRB2	FC	21.7±0.98^{a}	2.4±0.17^{a}	29.0±1.15^{a}	5.2±0.11^{a}	27.8±1.61^{a}
	FO	20.0±1.15^{a}	2.3±0.17^{a}	29.0±2.03^{a}	5.1±0.13^{a}	26.9±1.97^{a}

FC: fresh cultures, FO: formulation, N: Nitrogen, P: Phosphorus, K: Potassium, Mg: Magnesium, Ca: Calcium, WHC: Water holding capacity. Values are the mean with in the column sharing the same letters are not significantly different according to Tukey's HSD at $P \leq 0.05$.

Table 3
Effect of seed treatment with formulations of selected PGPR on Fusarium wilt and Early blight incidence under greenhouse conditions.

Isolates	Treatment	Fresh weight (g/plant)	Total chlorophyll (mg/g tissue)	Fusarium wilt incidence (%)	Early blight incidence (%)
Control		199.5±5.48^{b}	16.5±0.11^{a}	76±2.30^{a}	80±1.73^{a}
2apa	FC	230.6±3.23^{a}	16.5±0.17^{a}	14±0.00^{b}	19±1.15^{b}
	FO	230.0±5.36^{a}	16.5±0.08^{a}	12±1.15^{b}	17±1.73^{b}
PSIRB2	FC	240.3±4.53^{a}	16.7±0.40^{a}	74±3.46^{a}	81±3.46^{a}
	FO	239.3±3.06^{a}	16.7±0.17^{a}	72±4.04^{a}	80±3.46^{a}

FC: fresh cultures, FO: formulation. Values are the mean with in the column sharing the same letters are not significantly different according to Tukey's HSD at $P \leq 0.05$.

DISCUSSION

Several attempts have been made previously to prepare formulations of rhizobacteria [22] and also talc based formulation of specific strain of fluorescent *Pseudomonas* [23]. Previously, Kloepper and Schroth [13] demonstrated that the potentiality of talc as a carrier to be used for formulating rhizobacteria. The fluorescent Pseudomonads did not decline in talc mixture with 20% xanthum gum after storage for two months at 4° C. According to the report of Mathre *et al*.,[24] *Bacillus* spp. may be only genus of bacteria that meets the shelf life standard required by a commercial microbial product. In addition to long term viability, *Bacillus* spp. have become commercially successful due to their ability to effectively colonize plant roots, produce antifungal compounds and secrete volatile substances that can directly stimulate plant growth [25]. Being Gram –ve bacteria, isolate 2apa was unable to survive in talc powder for longer period. But, according to studies of Vidyasekaran and Muthamilan [14], *P. fluorescens* isolate Pf1 survived up to 240 days in storage. The initial population of Pf1 in talc-based formulation was 37.5 × 10^7 cfu/g and declined to 1.3 × 10^7 cfu/g after 8 months of storage. The bacterial formulations of talcum powder developed were evaluated for their plant growth promoting and disease protecting ability under greenhouse conditions. Among the isolates selected, the isolates PSIRB2 and 2apa were found to promoting the plant growth well under both laboratory and greenhouse conditions when compared to control. The isolate PSIRB2 was known to produce IAA and also solubilize the phosphate by producing organic acid and enzyme phytase [15] and isolate 2apa also characterized as a producer of IAA and siderophore [6]. These unique characters of these isolates were attributed to promote early growth of tomato plants. Indole acetic acid and phosphate solubilizing rhizobacteria were frequently being reported as PGPR [26,27,28,29]. Further, when formulations of these isolates were evaluated for their disease protecting ability, isolate PSIRB2 completely failed to protect tomato seedlings from both Fusarium wilt and Early blight diseases. Isolate 2apa significantly reduced the incidence of both root and foliar fungal pathogens of tomato studied. As previously studied, isolate PSIRB2 did not have any mechanism to inhibit fungal growth and was induced systemic resistance (ISR) negative [15]. Whereas isolate 2apa is known to produce an antibiotic – Phenazine with wide range of antimicrobial properties, along with the isolate can induce systemic resistance against foliar pathogen. Hence, the disease protection results revealed that these mechanisms of the selected rhizobacterial isolates are playing an important role in determining their disease suppressing ability under greenhouse conditions. The bottom line for biocontrol is whether it works under production conditions or not. Biocontrol agents are being tested more often in the production system for which they are intended, rather than relying solely on experiments done *in vitro*, on detached leaves, on plantlets, or in the greenhouse on non-greenhouse crops. Our findings are in accordance with that of Jayaraj *et al*.,[30] who attempted an integrated approach for damping-off management by employing dual inoculation of PfT-8 in seed and soil coupled with soil application of organic amendments including poultry manure or FYM under field conditions. They reported a significantly reduction of damping-off incidence up to 90% and further, significantly increased healthy plant stand, plant biomass and plant rhizosphere population of *P. fluorescens* in tomato. In conclusion, the talc formulation of biocontrol agent 2apa either alone or in combination with organic fertilizers can be recommended to the farmers as one of the crop protection strategies for the management of fungal diseases of tomato and its practice may also be extended to other *Solanaceous* crops. The strain *Pseudomonas aeruginosa* 2apa was originally isolated from rhizospheric soil sample of tomato and also found significantly improving the plant growth and suppressing fungal diseases of tomato [6]. Further work to understand its pathogenicity and multidrug resistance is under progress. Before taking into the field, further work will be finished. Further, we are also aiming our work to use organic manures instead of chemical fertilizers along with PGPR.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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Reviewers of this article

Dr.Mohammed Aiyaz

Research Associate, School of Biology, Indian Institure of Scientic Education and Research, Trivandrum - 695016, India

Mr. Anubrata Paul M.Sc. Biotech (Research)

Department of Biotechnology, Natural Products Research Laboratory, Centre for Drug Design Discovery & Development (C-4D) , SRM University, Delhi-NCR,Sonepat.

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