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COMPARATIVE SURVIVABILITY OF *PSEUDOMONAS FLUORESCENS* RRB 11 IN DIFFERENT CARRIERS AND RHIZOSPHERE

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ABSTRACT
The bioformulation of *Pseudomonas fluorescens* RRb-11 was prepared using talc powder, kaolinite powder, barley bran, wheat bran and soybean bran as carrier substrate for the management of bacterial leaf blight of rice using seed treatment as delivery method. The survival of *Pseudomonas fluorescens* RRb-11 was tested from bioformulation at every 30 days of storage. The maximum survivability of *P.fluorescens* was observed in talc based bioformulation even up to 150 days after storage. On all substrate the population of RRb-11 decline evenly but population reduced drastically after 120 days of storage. No survival of RRb-11 was observed in bioformulation of wheat and soybean bran from 60 days of storage. The survivability of RRb-11 was also enumerated in rhizosphere where the talc based bioformulation caused slow reduction in population density of RRb-11 as compare with other bioformulations. However, the population density of RRb-11 in rhizosphere reduced more drastically soon after delivering it in the soil as compare with the bioformulation kept in storage.

INTRODUCTION

Survival and establishment of inoculated strains in the rhizosphere in competition with native microbial flora is important. Several reports support the use of selected microbial antagonists for control of seed and soil borne phytopathogens but there are very few commercial products yet available. A key constraint to commercialisation is the availability of effective formulations and delivery systems that insure long term viability of the inoculant and its biocontrol activity (McIntyre and Press, 1991). Application of microbial antagonists, for example *Pseudomonas* spp., to seed provides an ideal delivery system as it introduces inoculum to the rhizosphere where plant pathogens such as *Xanthomonas*, *Pythium* and *Rhizoctonia* on seed and soil are active, causing blight, seed rots in the spermosphere and seedling damping-off. While bacteria can be applied directly to the seed surface, vegetative bacterial cells are very susceptible to the physical and chemical stresses associated with seed preparation, such as desiccation or temperature changes. The antagonistic bacterium released into the rhizosphere is considered as a mean to suppress phytopathogens. This approach requires a means of monitoring the organisms after their release. To be enumerated, a released organism must have a selective characteristic which does not interfere with its inherent ability to survive and colonise the environment. Commercial application of PGPR either to increase crop health or to manage plant diseases depend on the development of commercial formulations with suitable carriers that support the survival of bacteria for a considerable length of time. Carriers may be either organic or non-organic. In addition, seeds of many species need to be treated with additional material to ensure ease of handling and sowing. Carriers increase the survival rate of bacteria by protecting it from desiccation and death of cells (Heijnen *et al*., 1993). The shelf life of bacteria varies depending upon bacterial genera, carriers and their particle size. When the seeds treated with carrier based bioformulation sowed in soil there appears to be reduction in inoculum potential of the treated bacteria. Successful soil inocu-lation of microorganisms requires survival of the introduced strain in soil, which largely depends on the availability of the empty niche and the capacity of competing with the better adapted native microflora (Lugtenberg *et al*., 1999; Rekha *et al*., 2007). Understanding of abiotic factors which affect the colonisation of microorganisms in the rhizosphere of plant is of primary importance for the effective use of rhizobacteria as plant growth stimulators (Schroth and Becker, 1990). Earlier reports claim that soil salinity has an adverse effect on plant growth promoting bacterial populations by high osmotic strength (low water potential) and toxic effects by salts (Borneman *et al*., 1996; Sato and Jiang, 1996).

The characteristics of formulations used to deliver bacterial

seed inoculants can influence the subsequent behaviour of the inoculated bacteria in the rhizosphere (Moenne- Loccoz *et al.,* 1999). An important next step in the development of seed treatments that incorporate fluorescent pseudomonads will be to determine the effect of seed treatment on colonisation of the roots and protection of seedlings under disease pressure.

The present investigation is designed to inves-tigate the effect on the colonisation of selected plant growth-promoting bacteria *Pseudomonas fluorescens* RRb-11 in the rhizosphere of paddy and bioformulation in storage to determine their survival in carrier material and on the plant roots in field experiments.

MATERIALS AND METHODS

Plant material and PGPR strain (*Pseudomonas fluorescens* RRb-11) isolation and maintenance

Mahi Sugandha, a susceptible variety of rice to bacterial leaf blight pathogen *Xanthomonas oryzae pv. oryzae* (Xoo) was selected for the experiment. Isolation of rice rhizosphere bacteria (RRb-11) was made from twenty rhizospheric soil samples of Basmati rice grown in the field of IARI, New Delhi and Almora. Ten grams of rhizosphere soil was taken into a 250mL of conical flask, and 90mL of sterile distilled water was added to it. The flask was shaken for 10 min on a rotary shaker. One millilitre of suspension was added to 10mL vial and shaken for 2 min. Different dilutions of working samples were prepared by serially diluting the stock solution. An aliquot (0.1mL) of this suspension was spread on the plates of Nutrient agar (NA) medium. Plates were incubated for 3 days at 28ºC to observe the colonies of bacteria. Bacterial colonies were streaked to other NA plates and incubated at 28ºC for 3 days. Typical single bacterial colonies were observed over the streak. Well isolated single colony was picked up and re-streaked to fresh NA agar plate and incubated similarly.

Rhizosphere bacteria *P. fluorescens* (RRb-11) strain which was earlier characterized biochemically and identified as *Pseudomonas fluorescens* in our lab. It has been found to produce Hydrocyanic acid (HCN), siderophores and 2, 4-diacetylphloroglucinol, Phenyl alanine ammonia lyase (PAL) and Peroxidase (POD).

Development of bioformulation

Mass multiplication of Pseudomonas fluorescens (RRb-11) in various carriers

The formulation was developed with slight modification in a process as described by Amer and Utkhede, 2000 using different carriers. *Pseudomonas fluorescens* strain RRb-11 was grown in liquid Nutrient broth for 48h as shaker culture in shaker incubator at 150 RPM at 28ºC temperature. The carboxy methyl cellulose (CMC), carrier and bacterial suspension in broth (10^8 c.f.u. mL^1) were used in the ratio of 1:50:4 The bioformulation was prepared as: talc powder (5.0g carboxy methyl cellulose (CMC) + 250 g talc powder (autoclaved at 121ºC at 15 p.s.i. for 30 min) + 20mL of bacterial suspension in broth; Kaolinite powder (5.0g CMC + 250g autoclaved Kaolinite powder + 20mL of bacterial suspension in broth); Wheat bran (5.0g CMC + 250g autoclaved Wheat bran + 20mL of bacterial suspension in broth); Barley bran (5.0g CMC + 250g autoclaved Barley + 20mL of bacterial suspension in broth); Soybean bran (5.0g CMC + 250g autoclaved Soybean bran + 20mL of bacterial suspension in broth) and 20mL of Bacterial broth suspension alone as a control. The bioformulations were dried overnight aseptically at room temperature. The materials were stored in sealed plastic bags at room temperature. Three independent samples were analysed with three replications for each analysis.

Survival of *P.fluorescens* RRb 11 in various carrier based bioformulation

The survivability of *P. fluorescens* RRb 11 cells was determined in five bio-inoculant preparations, including Talc, Kaolinite, barley bran, soybean bran and wheat bran. Samples of 10 g were drawn at 0, 30, 60, 90 120 and 150 days after storage from four bags of each carrier material at each time and bacterial population was assessed by dilution plate method on nutrient agar medium under aseptic conditions. Suitable dilutions were spread plated on NA medium, amended with antibiotics (rifampicin and streptomycin-50 ìg/mL each), and incubated at 28 ± 1ºC for 48h. The bacterial population (CFU/g) was enumerated. The experiment was conducted in triplicate, and one bag of each carrier from each replicate was investigated after every 30 days interval.

Effect of seed treatment with carriers on % disease intensity and rhizosphere competence of *P. fluorescens* RRb-11 isolate

The experiment was conducted at Agriculture research Station, Banswara, Rajasthan in 2009 and 2010 kharif season. Seeds of Mahi Sugandha were surface sterilized with 1% sodium hypochlorite for 1-2 min. and washed and rinsed in sterilized distilled water (SDW) four times and dried overnight in shade. The rhizobacterial culture was separately grown in nutrient broth for 48h at 28ºC in shaker incubator (150 rpm). The broth obtained is dissolved in sterilized distilled water to obtain the population density of 10^7 $cfuml^{-1}$ (0.1 O.D. at 620 nm).The suspension was mixed with 2 % CMC and different carriers and treated the seeds at the rate of 5g/kg seeds. The seeds were allowed to dry overnight in aseptic condition after coating with carrier mixed bacterial culture and CMC. The treated seeds sown in the field. There were four replications for each treatment and the design was Randomised block design. The % Disease Intensity was recorded by following formula given by Jayaraman and Meena (2004).

$$(\%)\ \text{Disease Intensity} = \frac{\text{Leaf length - Lesion Length}}{\text{Leaf length}} \times 100$$

The bacterial population densities in the rhizosphere of rice plants, grown during field trials were analyzed at every 30 days intervals on NA supplemented with streptomycin and rifampicin (50ìg/mL each). Sampling involved uprooting plants from the field plot at every 30 days intervals. The root mass was placed in a universal bottle containing 10mL SDW (Sterile Distilled Water). The closely adhering rhizosphere soil was washed off by agitating the bottles by hand, and the roots were then removed from the universal bottle. The rhizosphere soil suspension was serially diluted and plated in triplicate onto Nutrient Agar medium amended with antibiotics, and incubated at 28 ± 1ºC for 48h to assay the population density of bacterial isolate RRb-11.

RESULTS AND DISCUSSION

Survivability of *P.fluorescens* in bioformulation of various carriers

The shelf life of environmentally sensitive microorganisms continues to be a challenging and success-limiting step in development of a bioformulation (Paau, 1998).The bacterium *Pseudomonas fluorescens* survived well in talc, Kaolinite and barley bran even up to 150 days after storage. But wheat and soybean bran did not support the growth after 60 days of storage. Among all the carriers tested, the bacterium survived best in talc powder (Fig. 1). This may be because talc has very low moisture equilibrium, relative hydrophobicity, chemical inertness, reduced moisture absorption and prevent the formation of hydrate bridges that enable longer storage period and these results are in strong agreement with previous findings of other researchers (Vidhyasekaran and Muthamilan, 1995; Vidhyasekaran *et al.,* 1997; Bora *et al*., 2004). The initial population of *P.fluorescens* was high in all the treatments which later on starts declining as the period of storage increases. The maximum survivability of *P.fluorescens* was observed in talc based bioformulation even up to 150 days after storage. The initial population of the bacteria was 310.8 $X10^7$ cfu/g and the population of 32 X 10^7 cfu/g was detected 5 months after storage at room temperature 27°C.Next best survivability of *P.fluorescens* was shown by Kaolinite based bioformulation which also persists up to 150 days of storage. Survival of *P. fluorescens* in talc, Kaolinite and barley bran having relatively smaller particle size which increased the survival rate than in wheat and soybean bran with bigger particle size. The carriers with smaller particle size have increased surface area, which increased resistance to desiccation of bacteria by increased coverage of bacterial cells (Nakkeeran *et al*., 2005).

Survivability (Rhizosphere competence) of *P. fluorescens* (RRb-11) in rhizosphere

P. fluorescens (RRb-11) isolate with different carriers applied successfully to seed to control *Xanthomonas oryzae* pv. *oryzae*. The bacterial isolate *P. fluorescens* RRb-11 tended to increase in number in rhizosphere soil over 45-60 days after treatment. RRb-11 survived well on roots or in the rhizosphere of Mahi Sugandha plants. This isolate was found to have an efficient colonization ability of 128.6 $X10^4$ cfu/g and 139 $X10^4$ cfu/g in seeds treated with talc based bioformulation at 45-60 Days after treatment in rhizosphere study. The root rhizosphere colonisation tended to increase population of *P.fluorescens* RRb-11 at increasing rate up to 60 DAT thereafter the population reduced and finally declined at 90 DAT, in all the treatments. But appreciably highest population of *P. fluorescens* RRb-11 was observed in talc based bioformulation treatment at 60DAT. According to Sarvanan *et al.,* 2004 who studied effect of *P.fluorescens* on fusarium wilt pathogen in banana rhizosphere, gradual increase in population of *P. fluorescens* in rhizosphere over 60 days after inoculation. Root rhizosphere of wheat and soybean based bioformulation treated plants showed lower rhizosphere population of *P. fluorescens* RRb-11. This may be due to inability of *P. fluorescens* RRb-11 to survive on wheat and soybean bran as found in shelf life study. But the root rhizosphere of talc, Kaolinite and barley based bioformulation treated plants showed good survivability of *P. fluorescens* RRb-11. Moreover, *P. fluorescens* RRb-11 maintained its population in rice rhizosphere even after 90 DAT. Molina (2000) studied the survivability of *Pseudomonas putida* KT2440 in soil and in rhizosphere and found that *P. putida* KT2440 established high cell densities in rhizosphere during crop growth of 12 to 16 weeks than in non planted soil where the number of cells was below detection limits after 50 days from initial cell density.

Disease intensity results were in concurrent with the results obtained in the survivability study. Thus it proves an established theory that all disease suppressive mechanism exhibited by *P. fluorescens* are essentially of no real value unless these bacteria can successfully establish in the rhizosphere. The maximum survivability of RRb-11 in rhizosphere was obtained in talc based bioformulation treated seeds. Similarly, when seeds were treated with various carrier based bioformulation of *Pseudomonas fluorescens* RRb-11in field conditions, the talc based bioformulation treatment showed minimum disease intensity of 8.47% and found to reduce disease intensity to the extent of 83.87% as compare with control. These results clearly supports survivability tests. The seed treated with Kaolinite based bioformulation exhibited disease intensity of 12.66% which reduce disease intensity by 75.9% as compare with control. The % disease intensity in untreated control was 52.6%.

In the present study the *Pseudomonas fluorescens* RRb-11 population in carrier based substrates declined markedly with storage time. Talc, Kaolinite and barley bran based bioformulation supported the bacterial population up to 90 days of storage and later the cell proliferation slowed down and slow cell death occurred. The population of cell was at par with the Bureau of standards in India as the carrier supported growth of 1 X 10^9 cfu/g up to 90 days of storage in talc, Kaolinite and barley bran formulation in storage. Survival of *P. fluorescens* (RRb-11) in talc and Kaolinite with smaller particle size increased the survival rate than in bran with bigger

Table: 1 Effect of different carriers on rhizosphere competence of *Pseudomonas fluorescens*

Treatment	Rhizosphere population at different days treatment					
	15 DAT 10^4 cfu/g	30 DAT 10^4 cfu/g	45 DAT 10^4 cfu/g	60 DAT 10^4 cfu/g	75 DAT 10^4 cfu/g	90 DAT 10^4 cfu/g
Wheat + RRb 11	66	29.2	88	81	29	18
Soybean + RRb11	43	38	83.1	69	41	14.5
Barley + RRb11	87.4	41	143	121.3	72	49.1
Talc + RRb 11	84.4	39	128.6	139	93	71
Kaolinite + RRb 11	65	28.2	117	103	78	47
Control	1.8	1.4	1.4	2.5	2	1.85
CD (P=0.05)	18.24	12.13	20.25	11.05	13.68	21.14

*Average of four replications; The initial number of cells for each treatment was 1 X 10^8 cfu/mL.; DAT: Days after treatment; Values presented in the table are average of two years

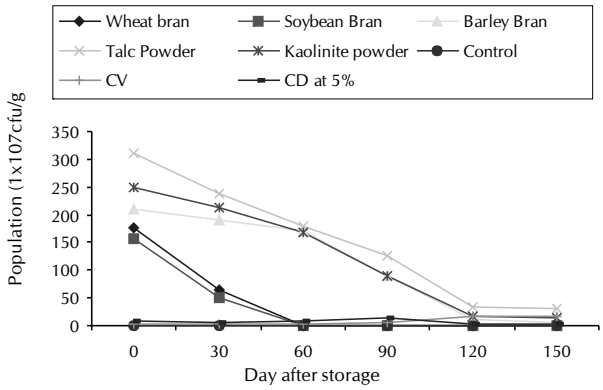

Figure 1: Survival of *P. fluorescens* in different carrier bioformulation kept in storage

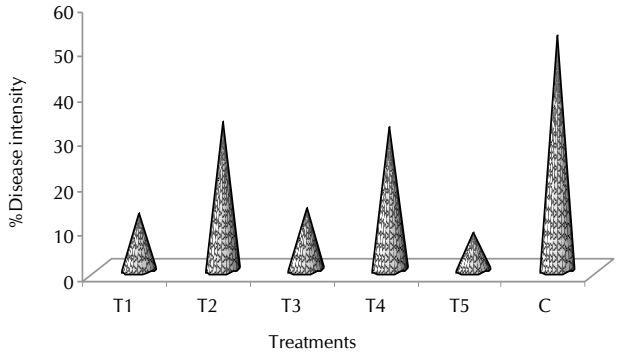

Figure 2: Effect of seed treatment with different carrier based bioformulation on disease intensity

particle size. The carriers with smaller particle size have increased surface area, which increase resistance to desiccation of bacteria by the increased coverage of bacterial cells (Dandurand *et al*., 1994). Persistence of *Pseudomonas* populations in soil has been monitored by several workers as part of studies on strains with biological control potential. For example, *P. extremorientalis* TSAU20 and *P. chlororaphis* TSAU 13 have the ability to survive in ecologically stressed conditions, such as saline and nitrogen deficient soils, and may positively effect on plant growth of bean. High sa-linity inhibited their colonisation in the rhizosphere of bean and thus their stimulatory effect on plants was also reduced (Egamberdieva, 2011). Similarly, Troxler *et al*. (2012) conducted experiment on persistence of inoculants of *Pseudomonas* in deep soil profiles. The results indicate that field-released *Pseudomonas* inoculants may persist at high cell numbers, even in deeper soil layers, and display a combination of different physiological states whose prevalence fluctuates according to soil microbial habitats.

In conclusion, the results of the study indicated that the maximum survivability of *Pseudomonas fluorescens* RRb-11 was found in talc based bioformulation both in storage and rhizosphere. It was observed that carriers with smaller particle size such as talc and Kaolinite powder and barley bran supported bacterial survivability more efficiently than the wheat and soybean bran with bigger particle size because smaller particle size have increased surface area, which increase resistance to desiccation of bacteria by the increased coverage of bacterial cells. Thus, survivability can be improved by using carrier substrates with smaller particle size. The rhizospheric survivability of *P. fluorescens* RRb-11 was found to reduce drastically soon after introducing it in the soil. Thus there is a need of further study to improve survivability of inoculum potential in the rhizosphere to manage the seed and soil borne phytopathogens.

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