Int.J.Curr.Microbiol.App.Sci (2019) *8*(8): 394-405

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 8 Number 08 (2019)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2019.808.045

Impact of Bacterization of *Rhizobium* and *Methylobacterium radiotolerans* on Germination and Survivability in Groundnut Seed

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Keywords

Bacterization, Groundnut, Methylobacterium, Rhizobium, vigour index, survivability

Article Info

Accepted:
04 July 2019
Available Online:
10 August 2019

A B S T R A C T

Groundnut contributes 40% of the total oil seeds production in the country. Groundnut is a rainfed crop in kharif season with least productivity. Hence an attempt was made for improving the abiotic stress resistance of plants through seed bacterization with biological agents. The present study was undertaken to examine the impact of bacterization of *Rhizobium* and *Methylobacterium radiotolerans* to enhance groundnut germination rate and vigour index. The results showed that groundnut showed higher response to TNAU 14 rhizobial strain, is a common feature with significant increase in germination percentage (100%) and vigour index (4098). The result of the present study indicate that root colonization ability of the phyllosphere bacterium *M.radiotolerans* individual strain had higher influence on the shoot length (24.1 cm plant^{-1}), root length (24.9 cm plant^{-1}) and seedling length (49.03 cm seedling^{-1}), when compared to co-inoculation and individual inoculation with *Rhizobium*. Seed survivability of bioinoculants after bacterization showed, *M. radiotolerans* had extended survival upto 196 h when compared to combined inoculation upto 96 h. The lowest survival was observed in *Rhizobium* treated seeds up to 72 h. Further the experiment also revealed that *M. radiotolerans* could have the ability in inducing the induced systemic resistance activity (ISR) in against groundnut seed borne fungal pathogen.

Introduction

Groundnut (*Arachis hypogaea* L.) is one of the most widespread and potentially most important food legumes in the world. India has the largest area under groundnut cultivation in the world but it ranks second in production, next to china. Peanut contributes about 40 per cent of the total oilseeds production in the country (Sathya Priya, *et al.*, 2013) and occupies first place in acerage (more than 28%). It is grown over an area of 5.31 million ha and producing 6.93 million tonnes of Peanut (DOAC, 2012) with productivity of 1305 kg/ha in Indian context. Its cultivation is mostly confined to the states of Gujarat, Andhra Pradesh, Maharashtra, Tamil Nadu and Karnataka. Groundnut is a rainfed crop in

kharif season and irrigated crop in rabi in some states (Varghese, 2011). In India, the rainfed groundnut ecology is the largest one in terms of area (8.0 Mha) and production (7.4 MT) but with least productivity (0.93 MT). These low yields are due to a number of biotic and abiotic constraints, such as lack of access to quality inputs, improved technologies and information, climate change and frequent attacks by pests and diseases.

One of the popular methods for improving the abiotic stress resistance of plants is introducing biological agents as seed treatment. Several studies have reported that 'pre-sowing seed treatments such as seed priming and seed coating could improve the seed germination and seedling growth. Seed coating is a technique in which various materials, such as fertilizers, nutritional elements, moisture attractive or repulsive agents, plant growth regulators, *Rhizobium* inoculum, chemicals and pesticides are added to the seed surface by adhesive agents (Scott, 1989). Studies have reported that seed coating could enhance germination, promote root and shoot growth and increase stress resistance (Hirano *et al.,* 2001). Applying microorganisms to seed is an attractive proposition because of the combination of specific effect and limited environmental impact. With increasing public awareness of the potential environmental and health hazards of both agrochemicals and fertilizers and the advances in biotechnology to improve the performance of microbial products, application of microorganisms to seeds is likely to increase in the future.

Microbes associated with a plant can have beneficial interactions, providing its partner organism biologically active compounds necessary for survival and proliferation (Marschner *et al.,* 2001). Mutualistic associations between root associated microbes and plants bring benefits to the plant through an increased nutrient acquisition, altered metabolic interactions among the partners, alleviation of salt stress and improved symbiotic performance of legumes. There is a possibility for selection of plant associated microbes from other plant habitats to broaden the spectrum of beneficial bacteria. Two general types of bacteria have been shown to have the capacity to act as growth promoting bacteria are; rhizospheric bacteria, that are typically found around the roots of plants; and endophytic bacteria (Lacava and Azevedo, 2013), that are found within the tissues of the plant itself. Mutualistic associations between plant associated microbes and its host plants provide several benefits to the plant through an increased nutrient acquisition, altered metabolic interactions among the partners, alleviation of stress and improved symbiotic performance of legumes. The endophytic lifestyle may directly or indirectly assist during the infection and colonization processes of the *Rhizobium*-host association and are coordinately involved in the adaptation of plants to stress tolerance (Hashem *et al.,* 2016, Egamberdieva *et al.,* 2016).

Bacteria belonging to the genus *Methylobacterium*, commonly known as pink pigmented facultative methylotrophic bacteria (PPFMs), which are ubiquitous on leaf surfaces and potentially dominating the phyllosphere bacterial community normally occur as rhizosphere inhabitants and endophytes when isolated on selective media (Corpe and Rheem, 1989).

Methylobacterium sp. impart beneficial effects on plant growth through direct or indirect mechanisms, which include production of phytohormones or enzymes that modulate plant growth, secretion of compounds involved in biocontrol, or disease suppression (Holland and Polacco, 1992 and Madhaiyan *et al.,* 2007).

However, despite the importance of the endophyte-plant relationship, our knowledge on the interactions between legumes, endophytes, and *Rhizobium* is still rather limited. In view of this, our aim of the present study was to determine the synergistic interaction between *Rhizobium leguminosarum* (TNAU 14), *Methylobacterium radiotolerans* (VRI8 A4) for its effect on groundnut seedling growth, survival in seed and vigor index when applied as seed treatment.

Materials and Methods

Plant material

Groundnut seed variety VRI8 was obtained from Regional Research Station, Tamil Nadu Agricultural University, Viruthachalam, Tamil Nadu, South India. It is a bunch type variety with initial germination of more than 95% and seed moisture content of below 10% was used in this study. It is medium bold kernel type with shelling percentage (70%), oil content (49%) and moderately resistant to late leaf spot and rust.

Microorganism

The selected *Rhizobium leguminosarum* strain (TNAU 14) was previously isolated from groundnut and *Methylobacterium radiotolerans* (VRI8 A4) previously reported by Krishnamoorthi *et al.,* (2018) was used in this study. Both strains were obtained from Department of Agricultural Microbiology, Agricultural College and Research Institute, Madurai, South India. *M. radiotolerans* (VRI8 A4) was isolated from VR18 a field grown bunch type groundnut variety from Virudhachalam, Tamil Nadu, South India. *M. radiotolerans* (VRI8 A4) possess multiple plant growth promoting traits like plant growth hormone synthesis (IAA), solublize minerals like phosphorus and zinc and sulphur oxidation, siderophore production and ACC deaminase and catalase activity. The microbial cultures were maintained in Yeast extract mannitol medium (YEMA) for *Rhizobium* and Ammonia Mineral Salt (AMS) medium supplemented with 0.5% methanol for *M. Radiotolerans.*

Culture preparation

The *Rhizobium* and *Methylobacterium* strains were cultured in YEMA and AMS broth respectively. The *Rhizobium* cells were incubated for 48h and *Methylobacterium* cells were incubated for 10 days to get a maximum cell population of 10^8 cfu ml^{-1}. The cells were harvested by centrifugation at 8,000 rpm for 20 minutes. The cells were resuspended in sterile phosphate buffer and adjusted to get a OD value of 1.0 at 600nm.

Seed Treatment

The surface of groundnut seeds were disinfected using 70% ethanol for 1 min followed by sodium hypochlorite solution (4%, v/v) for 2 min, and were then rinsed five times with sterile distilled water. Surface sterilized seeds were inoculated by imbibition with individual and combination of *R.leguminosarum* strain (TNAU 14) and *M. radiotolerans* (VRI8 A4). *R.leguminosarum* and *M. radiotolerans* strains cell population in liquid media was adjusted to 5×10^8CFU (colony-forming unit) ml^{-1}, obtained at exponential growth phase on YEM broth and AMS broth respectively. Inoculation doses were adjusted to equal volumes (1:1 ratio dilution) of each strain when used for co-inoculation. Surface sterilized seeds were immersed in respective bacterial suspension for an hour as individual and in combination. Four treatments were used: (1) non-inoculated seeds (control) treated with sterile distilled water; (2) seeds inoculated with *R.leguminosarum*; (3) seeds inoculated with

M. radiotolerans (4) seeds coinoculated with *R.leguminosarum* and *M. radiotolerans*.

Roll towel method (lSTA, 1985) was used to study the effect of *Rhizobium* and *Methylobacterium* strains as individual and combined inoculation on growth and vigor of the groundnut seedlings. In two moist germination paper towels of 23 X 30 cm size, 50 seeds per replication were kept at an appropriate spacing and covered with another moist paper towel and rolled up.

For each treatment five replications were maintained. The rolled paper towels were kept in an upright position in an incubator at 29±1°C (8 h light/16 h dark cycle) and a half-strength Hoagland's nutrient solution free of nitrogen was used alternatively to withstand the plants nutritional demand.

The germination counts were taken after 7 days and expressed as per cent germination and vigor indices were also calculated by following the procedure suggested by Abdul - Baki and Anderson (1973). At the end of 20 days, root and shoot length of seedlings, were recorded and the seedling vigor index 1 (VI-1) and seedling vigor index 2 (VI-2) were calculated using the formulae: VI-1=Percent of germination (%) × Seedling growth (shoot length + root length) (cm) VI-2=Percent of germination (%) × Seedling dry weight (g).

Shoot length and root length

Shoot and root lengths of the seeds were recorded on the basis of randomly selected 25 plants per treatment in lab condition (Dubey, *et al.,* 2011). The tip of the above ground trifoliate leaves as well as the root tip was tied in a single straight line and then the length was measured *i.e.* shoot length and root length. Observations were recorded on 20^{th} days of crop growth and expressed in cm per plant.

Dry Biomass of Plant

The dry weight of the seedling was measured on the basis of randomly selected 25 germinated seeds per treatments. Plants samples were placed in hot air oven at 75º C till the moisture gets removed and until constant weight is obtained. After that they were weighed to determine mean seedling dry mass (g) in each replication.

Seed Inoculation and Survival

Surface disinfected groundnut seeds were imbibed in the respective microbial cultures (1% v/v) as individual and coinoculation. Seeds were allowed to imbibe for an hour and air dried in shade for 30 min and kept at room temperature to minic farmers practices. The number of viable cells adhering to each seed was estimated at regular interval of every 12 hours. Bacteria survival was measured periodically by transferring 10g of bacterial imbibed seeds to 100 ml sterile phosphate buffer followed by 5 minutes agitation and the suspension was plated by 10-fold serial dilutions upto 10^5 dilutions on Congo-red YEM agar for *Rhizobium* and in AMS agar medium supplemented with 5% of methanol for *M.radiotolerans*. Colony counts were made after incubation at 30^0 C for 2 days and 7 days for *Rhizobium* and *Methylobacterium* respectively. The experiment was terminated as the seeds were cross contaminated by fungal pathogen at varying intervals depending on the treatment.

Statistical analysis

Statistical analysis was performed by subjecting the data to one way analysis of variance (ANOVA) and analyzing them by Tukey least significant difference test for statistical significance at $p \leq 0.05$ using SAS software, version 9.4 (SAS, Cary, NC).Where there was statistical significance (P = 0.05),

the mean values were further separated using the LSD.

Results and Discussion

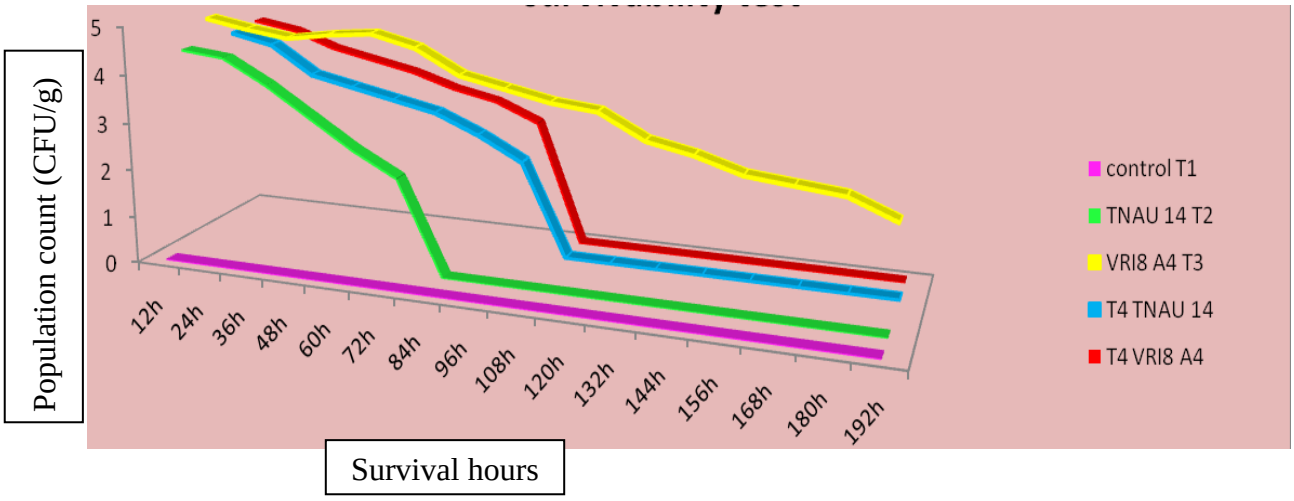
The most popular N_2-fixing plant-microorganism interaction is the legume–rhizobia symbiosis, which is considered as the most significant and efficient process in crop production to improve soil fertility (Mylona *et al.,* 1995). Improving the biological nitrogen fixation and nodulation in legumes by combined or co-inoculation of rhizobia with plant growth promoting microorganisms is recent strategy and a practical way of improving nitrogen availability in legumes (Abdel-Wahab *et al.,* 2008). Potentiality for improving plant yield by combining rhizobacteria with rhizobia has been reported by many workers (Pan *et al.,* 2002; Tilak *et al.,* 2006; Verma *et al.,* 2010). PGPRs with rhizobia can promote legume nodulation and nitrogen fixation is a synergistic interactions which are due to the synthesis of flavonoid like compounds, stimulating the host legume to produce more flavonoid signal molecules (Parmar and Dadarwal, 1999; Bai *et al.,* 2002), production of substances like hormones, siderophores, phosphate solubilization and /or improvement of nutrients and water uptake (Abdel-Wahab *et al.,* 2008, Verma *et al.,* 2010). The most commonly implicated mode to stimulate legume–*Rhizobium* symbiosis is phytohormones inducing stimulation of root growth, to provide more sites for rhizobial infection and nodulation (Vessey and Buss, 2002).

Members of the genus *Methylobacterium* are distributed in a wide variety of natural and man-made environments (Hiraishi *et al.,* 1995). In addition, methylotrophic bacteria are frequently associated with terrestrial and aquatic plants, colonizing roots and leaf surfaces (Corpe and Rheem, 1989; Lidstrom and Chistoserdova, 2002). The association of *Methylobacterium* species with plants seems to rely on a symbiotic relationship between the bacterium and the plant host. *Methylobacterium* species produce phytohormones (cytokinins and auxins), which are known to stimulate seed germination (Lee *et al.,* 2006), plant growth (Abanda-Nkpwatt *et al.,* 2006), fix atmospheric nitrogen (Sy *et al.,* 2001). Based on these previous reports we evaluated the combined inoculation of *R. leguminosarum* strain TNAU 14 and *M. radiotolerans* strain (VRI8 A4) under *in vitro* condition on the performance of groundnut seedling growth and vigor index. The results showed that positive crop response has been observed in all treatments of treated seeds either as an individual treatment or in combination when compared to uninoculated control. Results showed that groundnut response to TNAU 14 rhizobial strain is a common feature in increased germination rate than other treatments. The germination percentage as influenced by inoculation with *R. leguminosarum* strain TNAU 14 and *M. radiotolerans* strain (VRI8 A4) under *in vitro* condition when treated either individually or in combined inoculation represented in Table 1 showed seed germination and seedling vigor was significantly increased when compared to uninoculated control.

The influence of *R. leguminosarum* strain TNAU 14 and *M. radiotolerans* strain (VRI8 A4) under *in vitro* condition when treated either individually or in combined inoculation indicated irrespective of bacterial treatment, the groundnut seeds showed increased germination, shoot and root length, and seedling vigor I and II (Table 1) when compared to untreated seeds except dry matter production which has no impact within the treatments. In the initial stage of seed germination, *R. leguminosarum* (TNAU-14) isolated from groundnut nodule treated seeds

was found to be significantly effective in inducing maximum germination per cent (100%) followed by combined inoculation (90%) and *M. radiotolerans* treatment (80%). Overall germination percentage ranges from 75% in uninoculated control to 100% in *Rhizobium* treated seeds. We analyzed the induction of germination by the phyllosphere bacteria *M. radiotolerans* indicates that the habitat adaptation had little influence. Hence, an increased germination was observed over uninoculated control. The increased germination percentage, plant growth and yield in groundnut and tomato was reported by (Madhaiyan *et al.*, 2006 Senthilkumar and Krishnamoorthi, 2017). Earlier studies have also reported the beneficial effects of application of methylotrophic bacteria through seed imbibition (Holland and Polacco, 1994) in increasing soybean plant growth and productivity. Inoculation of seeds with beneficial biological agents in combination with priming (Biopriming) potentially able to promote rapid and more uniform seed germination and plants growth (Moeinzadeh *et al.*, 2010). But as we compared the growth of the seedlings, the single strain inoculation with *M. radiotolerans* had greater influence on the shoot and root length. The shoot and root length had increased by 41% and 12.6% when compared to combined inoculation 21.1% and 1%. It was also observed that the *M. radiotolerans* had greater influence in improving the seedling growth especially root growth (14.7%) as a plant growth promoting bacteria than the Rhizobium treated seed (0.4%). The results of the present study indicate the root colonization ability of the phyllosphere bacteria *M. radiotolerans*. Root colonization trait by PGPR is critical in plant growth promotion (Kloepper and Beauchamp 1992) and is highly dynamic process which is constantly affected by the various environmental factors (Weller 1988). The bio-priming of seeds by native methylotrophic bacterial isolates with single inoculation as well as combined inoculation (consortium) had significant role on seed germination, root length, shoot length and seedling vigor index of seedlings compared to other individual culture inoculation in rice (Ronak *et al.*, 2017).

Fig.1 Survivability of *Rhizobium* and *Methylobacterium* in groundnut seed



T_1- un-inoculated control, T_2- *Rhizobium leguminosarum* (TNAU 14), T_3- *Methylobacterium radiotolerance* (VRI 8 A4), T_4- Combined inoculation (TNAU 14 + VRI8 A4).

Table.1 Effect of seed imbibition with *M. radiotolerans* (VRI8 A4) alone or in combination with *R.leguminosarum* (TNAU 14) on groundnut germination and vigor index under *in vitro* condition

Treatment	Germination[a] (%)	Seedling parameters				Vigour index-I	Vigour index-II
		Root length (cm seedling^{-1})	Shoot length (cm seedling^{-1})	Seedling length (cm seedling^{-1})	Dry matter (g seedling^{-1})		
Control (T_1)	75±0.54^{d}	22.1±0.16^{c}	17.0±0.10^{d}	39.79± 0.33^{c}	0.37±0.0	3183.2±31.4^{d}	24.82±0.14^{d}
TNAU 14(T_2)	100±1.50^{a}	22.2±0.23^{c}	19.5±0.13^{c}	40.98±0.7^{c}	0.36±0.0	4098±22.3^{a}	36.88±0.21^{b}
VRI8 A4(T_3)	80±0.73^{c}	24.9±0.03^{a}	24.1±0.2^{a}	49.03±0.5^{a}	0.33±0.0	3677.2±6.25^{c}	29.76±0.22^{c}
Combined inoculation (T_4)	90±0.31^{b}	22.3±0.15^{b}	20.6±0.23^{b}	42.93±0.6^{b}	0.42±0.0	3863.7±6.57^{b}	37.80±0.1^{a}
Mean	86.25	22.81	20.32	43.2	0.37	3706	32.31
SEd	1.26	0.22	2.99	0.79	NS	28.10	0.23
CD=0.05	2.75	0.48	6.50	1.72	NS	60.98	0.52

[a]Values are the mean of 4 replicates of 50 seeds each.

Each value represents mean ± SE of 4 replicates treatment. In the same column, significant differences at $p \leq 0.05$ are indicated by different alphabets. Data followed by same alphabetic letter in the same column are not significantly different from each other.

LSD, Tukey least significant difference test.

The results showed that the inoculation of the groundnut seeds with *Rhizobium leguminosarum* (TNAU-14), significantly increased vigour index compared to other treatments (Table 1). The effects of plant growth-promoting rhizobacteria, including the production of the plant growth regulators, such as gibberllic acid, cytokinin and auxin, can directly and indirectly provide the favorable conditions for plant growth (Nadeem *et al.,* 2014). Increase in root length and shoot length of peanut seedlings may be attributed to the production of such growth regulators. Advancement in germination coupled with improved vigour index due to biopriming with *Rhizobium* at 20% for 12h with maize (Kalaivani *et al.,* 2010) and cluster bean (Ramamoorthy *et al.,* 2000) have already been reported. Inoculation of *Rhizobium* in consortium with free-living rhizospheric bacteria has also given excellent results in improving crop growth and productivity (Kishore *et al.,* 2005). Most of the *Rhizobium* species have been found to produce indole acetic acid (Ahemad *et al.,* 2012), which is essential for process of nodule formation through cell division and differentiation along with vascular tissue formation (Ahemad *et al.,* 2014).

The shoot and root length of seedlings by *M. radiotolerance* (VRI8 A4) treatment were significantly greater than the control (untreated). The bacteria, by increasing the root length and increased the plant's access to nutrients and water, thereby attracting the plant nutrients. *Methylobacterium spp*. significantly increased the seedling length of red pepper and tomato (Madhaiyan *et al.,* 2006; Ryu *et al.,* 2006). Bio-priming of tomato seeds with isolated *Methylobacterium extorquens* MM2 recorded higher seed quality parameters (Pattnaik Subhaswaraj *et al.,* 2017). IAA production by methylotrophic bacteria is directly correlated with increased root and shoots length. Recently, IAA producing phyllosphere methylotrophic bacteria in mustard leaves has been reported to improve tomato shoot and root length compared to control (Subhaswaraj *et al.,* 2017). In this study the result revealed that *M. radiotolerance* (VRI8 A4) has the highest ability to stimulate seedling growth (root length - 24.9 cm seedling^{-1}, shoot length-24.19 cm seedling^{-1} and seedling length 49.03 cm seedling^{-1}) compared to other strains.

The survival of viable cells of the *Rhizobium* and *Methylobacterium* on the seed is important criteria which decide the nodulation, nitrogen fixation, shoot and root elongation and mainly protect in the biotic and abiotic affect. The survivability of *Rhizobium* and *Methylobacterium* treated seeds were periodically checked for 9 days at 12 hrs intervals. When treated groundnut seeds were analysed for the survival of bioinoculants, there was no significant differences were observed in the population of *Rhizobium* and *M. radiotolerance* treated seeds upto 24 hrs irrespective of the treatment (Fig. 1). The soybean seeds after inoculation with *B. japonicum* when analyzed showed no significant differences (within 1–2 hours) (Stefan Martyniuk *et al.,* 2016). In general a gradual decline in the population was noticed in all treatments with increase of time. The optimum cell population in the seed after seed treatment was 10^4 as reported by Nambiar *et al.,* (1983). Results of previous studies have shown that good-quality rhizobial inoculants should provide about 10^3–10^5 of bacterial cells on each seed (Thies *et al.,* 1991; Singleton *et al.,* 1992). The present study shows a one fold higher population (10^5) of *Rhizobium* was observed.

Survivability of *M. radiotolerance* was more in treated seeds when compared to *Rhizobium*. It was also observed that the *M. radiotolerans* had extended survival (upto 192 hrs) when compared to combined inoculation (upto 96

hrs). The lowest survival was observed in the *Rhizobium* treated seeds (upto 72 hrs). *M. radiotolerance* were responded well to penetrate into the seed and retained the sufficient populations for 8 days at room temperature (10^5 cfu g^{-1} of seed) when compared to combined inoculation for 4 days and 3 days for *Rhizobium* treated seeds. There was a slow decline of one order of magnitude with respect to the initial population. Therefore, seed soaking in liquid microbial culture has the benefit of penetration and survival in the seed. *R. leguminosarum* when coinoculated with *M. radiotolerance* shows higher survival (12 hrs to 96 hrs and 4.3 x 10^4 to 2.0 x 10^4 cfu g^{-1} of seed) than the seed treated with *R. leguminosarum* alone (4.3 x 10^4 to 2.0 x 10^4 cfu g^{-1} of seed). However, the combined inoculation of rhizobial and *Methylobacterium* showed a marked increase in survival of the *Rhizobium* on such seeds may be due to possible synergistic effects of *Methylobacterium* sp. but further research is needed to study the survival bioinoculants under different environmental conditions and field effectiveness of this seed treatment process has to be validated.

Further the experiment also revealed that the *Rhizobium* treated seeds and uninoculated control seeds were infected by fungi after 72h (3 days after treatment) of storage at room temperature followed by combined inoculation of seeds showed fungal infection after 96h (4 days after treatment). Whereas the *Methylobacterium* alone treated seeds showed the fungal infection after 192h (8 days after treatment). The results indicates that *Methylobacterium* could have the ability in inducing the induced systemic resistance activity (ISR) in seeds after seed treatment. This is the first report of ISR activity of *Methylobacterium* in pretreated seeds against seed borne pathogen infection. We speculate that the *Methylobacterium* could produce chemical substances that might induce the defense related proteins and phenolics that could protect the seeds from seed borne pathogen infection. The ISR activity in rice and groundnut plants in response to methylotrophic bacteria suggests the possibility that PPFMs might be used as a means of biological control of disease was reported by Madhaiyan *et al.*, (2004 and 2006).For large scale adoption of this technique required detailed studies of *Methylobacterium* induced defense system in pretreated seeds before sowing.

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How to cite this article:

Priya, M., K. Kumutha and Senthilkumar, M. 2019. Impact of Bacterization of *Rhizobium* and *Methylobacterium radiotolerans* on Germination and Survivability in Groundnut Seed. *Int.J.Curr.Microbiol.App.Sci.* 8(08): 394-405. doi: https://doi.org/10.20546/ijcmas.2019.808.045