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Review Article

IMPACT OF CARRIER BASED ENDOPHYTES ON PLANT GROWTH

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ABSTRACT

Endophytes are mutualistic microbes that are naturally associated with plants. In non-leguminous plants, the endophytic bacteria enhance the plant growth and improve their nutrition through nitrogen fixation, phosphate solubilization or siderophore production and also produce phytohormones such as auxin, gibberellins, and cytokinins. Endophytic bacterial strains also increase the growth parameters such as height, girth, number of leaves and physiological parameters viz. chlorophyll stability index, stomatal resistance. The carrier based microbial inoculants enable long-term storage, easy handling and cost effectives of bio fertilizers. Some of the tested and acceptable carrier materials are bagasse, sugarcane filter mud, coir dust, coal, lignite, charcoal, straw and clay. Using biogas sludge as carrier, *Enterobacter* and *Bacillus* strain showed significant increase in root and shoot dry weight, length and seed weight. Bagasse, saw dust, wood ashes, coriander husk are low cost, ecofriendly and also support the high number of PGPR and maintain its survivability due to high moisture holding capacity. The physical and chemical characteristics of carrier material such as water holding capacity, organic matter, bulk density and porosity influence the growth of plants. Carrier material also plays an important role in maintaining shelf-life of microbes.

Keywords: Endophytes, Shelf-life, Bagasse, Bulk density

1. INTRODUCTION

Endophytes are mutualistic microbes that are naturally associated with plants. Most common endophytes include fungi and bacteria [1, 2]. The Endophytes are living in the internal plant tissues where they spend their complete or a stage of the life cycle. They don't cause any apparent or immediate disease symptoms [3-5]. This non-pathogenic association could be beneficial, neutral or detrimental [6]. Endophytic bacteria naturally occur as a result of plant growth or through root hairs and at epidermal conjunctions and also it can enter into the plants mainly through wounds. The wounds allowing leakage of plant exudates feed the bacteria [3]. Endophytic bacteria also enter through flowers, stomata and lenticels [7] (Table 1).

Table: 1 Endophytes in different plants

S.No	Plant Species	Endophytes	Reference
1.	Rice (Oryza sativa L.)	*Rhizobium leguminosarum*, *Azorhizobium caulinodans*, *Sphingobacterium sp. Serratia sp.*	[8-11]
2.	Potato (*Solanum tuberosum* L.) tuber	*Actinomyces, Agrobacterium, Alcaligenes, Arthobacter, Bacillus, Capnocytophaga*	[6, 12]
3.	Citrus plant	*Bacillus sp, Nocardiasp*	[13]
4.	Sugarcane (*Saccharum officinarum* L.)	*Herbaspirillum rubrisubalbicans, Acetobacter*	[14, 15]

2. POTENTIAL OF ENDOPHYTIC BACTERIA AS BIOFERTILIZER

In non-leguminous plants, the endophytic bacteria enhance plant growth and improve their nutrition through nitrogen fixation, phosphate solubilization or siderophore production (iron chelation) [5, 16]. It also produces phytostimulators such as phytohormones, the cofactor pyrroloquinolinequinone (PQQ) propane -1-

carboxylate (ACC) deaminase which enhance the plant growth and developmentby lowering plant ethylene levels and endophytes act as biological control agent against diseases [5, 17]. Endophytic bacterial strains also increased the growth parameters such as height, girth,number of leaves and physiological parameter viz., chlorophyll stability index stomatal resistance and transpiration in banana plants both under greenhouse and field conditions [18].

2.1. Biological Nitrogen Fixation

Nitrogen-fixing endophytic bacteria have been isolated from several groups of plants [19]. It includes *Gluconacetobacterdiazotrophicus* previously known as *Acetobacterdiazotrophicus* from Brazilian variety of sugarcane [14]. Its association with plant facilitates the plant growth [15].

2.2. Phosphate Solubilization

Endophytic bacteria are capable of solubilizing the unavailable form of phosphorus into available form thus promoting its uptake by plants [20].The most powerful strains of phosphate Solubilizers are *Pseudomonas, Bacillus, and Rhizobium*.

2.3. Siderophore Production

Endophytic bacteria produce siderophore (Greek "iron carrier"). They are low-molecular compound with high iron chelating affinity [21] and therefore improve the iron nutrition of plants [22].

2.4. Endophytes as Biocontrol Agents

Endophytes control plant diseases [23] by the production of antifungal or antibacterial agents [16, 17].

3. CARRIER MATERIALS

Carrier based inoculants containing effective microorganism is used as biofertilizer. It enables easy-handling, long term storage and high effectiveness of biofertilizers [24]. For seed or soil inoculation,various type of materials are used as carrier. For the purpose of seed inoculation the carrier material should be milled to fine powder with particle size of 10-40µm [25]. The characteristics of an efficient carrier material includes, non-toxic to inoculant bacterial strain, easy to sterilize, good absorption capacity, non-toxic to plant, low in content of soluble salts [26].

4. STERILIZATION OF CARRIER MATERIAL

To keep high number of inoculant bacteria on carrier for long storage period, sterilization of carrier material is essential. Gamma radiation is a method of sterilization, it make no chance in physical and chemical properties of the carrier material. It is packed in thin-walled polyethylene bag and then gamma irradiated at 50KGY(5µrads).Another method is autoclaving. The carrier material is packed in partially opened, thin-walled polypropylene bags and autoclaved for 60min at 121°C. During autoclaving, some materials change their properties and produce toxic substances to some bacterial strains [27] (Table 2).

Table: 2 Examples of Carrier Material [28]

S.No	Carrier Material	Bacterial Inoculum	Characteristics
1.	Composted sawdust	*Rhizobium , Azospirillum*	Good growth and survival of the inoculant strains.
2.	Wastewater sludge	*Sinorhizobium*	Seed inoculant
3.	Wheat bran, sugarcane bagasse	*Bradyrhizobium and phosphate solubilizing fungus A.niger*	The number of microorganism was highest with peat, followed by bran and sugarcane bagasse
4.	Peanut oil or soybean oil	*Rhizobium*	Seed inoculants
5.	Perlite	*Bradyrhizobium*	Combination of a sucrose adhesive with the perlite carrier gave better survival of bacteria on seeds.

The tested and acceptable carrier materials are bagasse, sugarcane filter mud, coir dust, coal, lignite, charcoal, straws, various compost mixtures, clays and minerals such as apatite and vermiculite. To support large populations of *rhizobia* over a long period of time, the choice of a carrier material is important. This will depend upon the physical and chemical characteristics of various carrier materials (Table 3). The sedge peat, charcoal and lignite have high water holding capacity.

Table: 3 Physical and chemical characteristics of various carrier material

S.No	Carrier	Organic Matter	Total %	Bulk Density G/Cc	Porosity%	Water Holding Capacity
1.	Sedge peat	76	0.95	0.82	45	200
2.	Farmyard manure	79	0.93	0.79	55	153
3.	Filter press mud	76	0.83	0.75	56	155
4.	Compost	55	0.55	0.75	59	171
5.	Vermiculite clay	1	0.01	0.98	63	152
6.	Lignite	75	0.31	1.08	35	198
7.	Charcoal	20	0.01	0.43	73	200

5. VARIOUS CARRIER MATERIAL USED IN ENDOPHYTIC BIOFERTILIZER AND THEIR CHARACTERISTICS:

The application of carrier material along with the bacterial inoculants has long been practiced [29]. Using biogas sludge as carrier, *Enterobacter strain 77-NS5, PSB12 and PSBS (Bacillus)* showed significant increase in root, shoot dry weight and seed weight. Most commonly utilized carriers are bagasse, sawdust, wood ashes, coriander husk etc. They support the high number of PGPR and maintain the survivability due to high moisture holding capacity, low of cost and environment friendly. Carrier materials such as karnolite, peat are high cost and environmentally unfriendly [22].

The carrier materials like coriander husk showed the best result with highest viable cell count in normal temperature over after the storage time of five weeks, but the other carrier materials showed a decline for room temperature. The coriander husk has high water holding capacity and so it remains the best to be used in biofertilizer [30].

The locally available organic manures such as aquatic weed (*Eichorniacrassipe*), coir dust, biochar and compost are tested for their performance as carrier materials for microbial inoculum. Those materials contain certain amount of major nutrient such as N, P and K [31] and were the best carrier for microbial inoculums [32] (Table 4).

Table: 4 Characteristics of different carrier materials

Carrier Material	pH	Water Holding Capacity (%)
Aquatic weed (*Eichhornia crassipes*)	8.1	311.5
Coir dust	6.8	298.5
Bio char	8.4	420.8
Compost	8.0	280.5

Biochar consists of a porous structure, so it has the ability to attract and retain water because of its high surface area and retaining its nutrients such as nitrogen and phosphorous. This is beneficial for growth of bacteria and fungi. Aquatic weed have the same C/N ratio of cow dung. Compost has microporous structure and they contain soil aggregates which will be good carrier for soil inoculants [33]. The plant growth promoting microorganisms isolated from the plants, use wood derived biochar as a potential carrier for the bioformulation on plant growth. The length of shoot and roots increased by 46% and 32% in comparison with control. The different carrier such as talc and charcoal based formulation of *Bacillus* sp on plant is effective than other carrier for promoting plant growth [34] (Table 5).

Table: 5 Effect of carrier based inoculum on physical parameters

Sample	Root Length(cm)	Short Length(cm)	Biomass(gm)
Control	17.37±0.56	35±0.45	22.17±0.47
Charcoal based formulation	22.83±0.52	3.53±0.67	31.58±0.89
Talc based formulation	20.66±0.47	42.04±0.14	26.62±0.75

The corn cob grits (waste product of corn plant in powdery form) are effective carrier material used for the biofertilizer preparation, because they are organic & naturally sourced. They are highly absorptive, non- toxic, reusable, biodegradable, high water holding capacity and also can absorb hazardous waste [35].

5.1. Test procedure for water holding capacity of carrier materials

One gm carrier was weighed in petridish and immediately placed in hot air oven at 105°C for 24 hrs and measured dry weight (gravimetric method for moisture content) with glass petriplates and analyzed the moisture content using formulation. For each carrier, this method was repeated for in triplicate [30].

5.2. Test procedure for total viable cell count of inoculated PGPR in formulation:

Experimental conditions

Ten gm of the carrier materials was taken into small disposable glasses and mixed well. Each glass of sterilized carrier materials inoculated with R*hizobium, Pseudomonas,* consortium of both bacteria in triplicate ($>10^8$cells ml^{-1}). According to the water holding capacity (checked by adding water in 10g of carrier), bacterial suspension was aseptically mixed with carrier in the following ratio:

- Sand (control), sand + *RH24* inoculum, sand + *pf23* inoculum, sand + *consortium* inoculum.
- Saw dust (control), sawdust+ *RH24* inoculum, sawdust + *pf23* inoculum, sawdust + *consortium* inoculum.
- Bagasse (control),bagasse + *rhizobia* inoculums, bagasse + *pf23* inoculums, bagasse+ *consortium* inoculum.
- Wood ashes (control), wood ashes + *consortium* inoculums.
- Coriander husk+ *Pseudomonas* inoculum, coriander husk + *consortium* inoculum.

For each treatment, three replicates were prepared in small polythene bags, where each glass contained 10g of carrier materials. For viable cell count (CFU g1) the selected carrier materials were placed for 5 weeks properly [30].

5.3. Total viable cell count

One g of sample for each glass was taken in test tubes and added 9ml of sterile distilled water and mixed thoroughly to ensure complete separation of bacteria from the carrier and serial dilution was performed (until10^{10}). Two drops of 10µl from each dilution was spread on minimal salt agar with two replicates for each dilution. All the plates were incubated at 28±2°C for 24 hrs and counted the colonies.

6. TEST PROCEDURE FOR DETERMINING SHELF-LIFE OF MICROORGANISM IN CARRIER MATERIAL

6.1. Enumeration of A*zospirillum lipoferum*

By using semi solid nitrogen free bromothymol blue medium, the population of *A. lipoferum* in both lignite and vermicompost carrier was enumerated. The MPN technique confirmed the formation of thin pellicle (3-5mm) below the surface of the medium [36]. Semisolid NFB medium was dispensed 5ml quantities in test tube and were sterilized. The 8th, 9th and 10thdilution of sample were used for enumeration of population. One ml of each sample was transferred to semisolid NFB medium contained in test tube with 5 replicates. For 5-15 days the tubes were incubated at room temperature. The development of sub surface pellicle and also change of the color of the medium from light yellow to blue was observed. The population of *Azospirillum* was calculated by using MPN table [36].

6.2. Enumeration of *B. megaterium*

The viable count of *B. megaterium* was done by plating technique using hydroxyl apatite agar medium by confirming the formation of solubilizing zone around the bacterial colony. Serial dilutions of inoculated sample were placed on hydroxyl apatite medium containing insoluble phosphate compounds. For 3-5 days, plates were incubated at room temperature. *B. megaterium* solubilize the insoluble form of phosphorus and shows clear zone around the bacterial colony [36].

6.3. Enumeration of *P. fluorescence*

The viable count for *P. fluorescence* was done by plating technique using Kings B agar medium by confirming fluorescent colonies on agar plate [36].

6.4. Population Calculation

Number of colony forming unit (CFU) per gram of sample is equal to the product of mean number of colony forming units and dilution factor by quantity of sample at dry weight basic.

6.5. Survival of *Azospirillum lipoferum*

The survival capacity of *A. lipoferum* in sterilized vermi compost carrier was recorded. It have maximum of 24.66 ×108 cfu/g of dry weight from 60th day to 120th day and then declined to 00.61 × 108cfu/g of dry wet on 360thday of the storage. Similar growth pattern is recorded when lignite is used as carrier material [37].

6.6. Survival of *Bacillus megaterium*

The survival capacity of *B. megaterium* on lignite carrier was recorded. It increases growth to 48.0×108cfu/g of dry weight on 90th day and then declined gradually to the value of 00.09×108cfu/g of dry weight on 360th day. But

when using vermicompost as carrier, the growth rate increased gradually from 28.33×108cfu/g of dry weight on 25th day to 71.0×108cfu/g of dry weight on 150th day and then declined gradually to 7.60×108cfu/g of dry weight on 360th day. So it was concluded that vermicompost carrier is better one for the survival of *B. megaterium* [37].

6.7. Survival of *Pseudomonas fluorescence*

The *P. fluorescence* in lignite as carrier material results in growth up to 90th day (43×108cfu/g of dry weight) and then gradually declined from 120th day to 360th day (22.70×108cfu/g of dry weight and 0.02×108cfu/g of dry weight). In the case of vermi compost as carrier, the growth gradually increased up to 90th day (49.23×108cfu/g of dry weight) and then decreased (5.00×108cfu/g of dry weight) on 360th day [37].

7. TEST PROCEDURES

7.1. Determination of organic matter content

To 0.1 to 2 g dried soil, added 10 ml of 0.167M $K_2Cr_2O_7$ and 20 ml of concentrated H_2SO_4 by avoiding excess swirling. Allowed to stand for 30 minutes, the suspension was diluted with 200 ml water (for clear suspension) and added 10 ml of 85% H_3PO_4 and 0.2 g NaF. To this added 10 drops of ferroin just prior to titration. The titration was done with 0.5 M Fe_2^+ to a burgundy endpoint. In the beginning the color of the solution was yellow orange to dark green, which shifted to a turbid gray and finally changed sharply to wine red at the endpoint. The reagent without soil can be used as blank [38].

7.2. Determination of bulk density

Bulk density of the sample can be determined by using core method. In core method, first the weight of the measuring cylinder is measured and noted. Then the oven dried sample is taken in that measuring cylinder and weighted. Note that, volume of the cylinder is same as total volume of sample ($\pi r^2 h$).Therefore bulk density is determined by dividing weight of the oven dried sample by total volume of sample [39].

7.3. Determination of porosity

Calculation from particle density and bulk density

The ratio of the bulk density, Pb to the particle density Pp, describes the fraction of the total volume occupied by solids [39]. Total porosity ϕ, is therefore calculated as,

$$\phi = 1 - (Pb/Pp)$$

7.4. Determination of NPK

7.4.1. Determination of nitrogen

7.4.1.1. Sample treatment

It is prime duty that all samples to be pre-treated to comply with the standard in the field of soil. It is necessary to maintain the temperature less than 400°C to avoid the loss of nitrogen during digestion [40]. Firstly, the homogeneity of the laboratory sample as well as the test sample was guaranteed.

7.4.1.2. Digestion

Dried and grinded sample portion of 0.2 gram (expected nitrogen content equal to 0.5%) to one gram (expected nitrogen content approximately 0.1%) was used. To this 10 ml sulphuric acid was added and swirled until the acid was thoroughly mixed with the sample. The mixture was allowed to stand for cooling. Then 2.5 g of the catalyst mixture was added and heated till the digestion mixture became clear. The mixture was boiled gently for 5 hours to allow the sulfuric acid condense about 1/3 to the end of the tube. The temperature of the solution was maintained below 400°C.

7.4.1.3. Titration

After the completion of digestion, the tube was left to cool and 20 ml of water was added by slow shaking. Then the suspension was transferred to the distillation apparatus. Then 5 ml of boric acid was added to a 200 ml conical flask and placed under the condenser of the distillation apparatus in such a way that the end of the condenser dips into the solution. Then 20 ml of sodium hydroxide was added to the funnel of the apparatus and ran the alkali slowly into the distillation chamber. Thereafter, about 100 ml of condensate was distilled and rinsed the end of the condenser, then few drops of mixed indicator were added to the distillate and titrated with sulfuric acid to a violet endpoint. Steam distillation was used. Distillation was stopped when 100 ml of distillation was collected.

7.4.1.4. Calibration

Calibration substances with known and unchangeable content of nitrogen were used to control the digestion and the apparatus. Sulfonyl acid with known nitrogen content was used. Besides these substances certified reference materials were used to control the whole procedure.

7.4.1.5. Blank determination

Two blank determinations were carried out in each series and the average blank value was used for subsequent calculations.

7.4.1.6. Quality Assurance of the overall procedure

*Duplicate determination***:** From the submitted sample for analysis, two sub-samples were tested. Control limit for differences between the results of the two sub sample was established, and precision was determined.

7.4.1.7. Expression of results

Method of calculation: The content of nitrogen (wN) in milligrams per gram was calculated using the formula:

$$wN = (V_1 - V_0) \times c\ (H+) \times MN / m \times mt \times 100$$

Where:

V1 is the volume in millimeters of sulfuric acid (4.7) used in the titration of the sample.

V0 is the volume in milliliters, of the sulfuric acid (4.7) used in the titration of the blank test

c (H+) is the concentration of H+ in the sulfuric acid (4.7) in moles per litre

MN is the molar mass of nitrogen in grams per mole (=14)

m is the mass of test sample

mt is the dry residue, expressed as g/100g on the basis of oven dried

7.4.2. Determination of Phosphorous in the laboratory

To extract phosphorus, well shaken 1g of air dried soil in 10 mL of 0.025 M HCl and 0.03 M NH_4F, for 5 minutes was prepared. Phosphorus was determined on the filtrate by the molybdate-blue method using ascorbic acid as a reluctant. Colour development was measured at 880 nm on a Brinkmann PC 800 probe colorimeter [40].

7.4.3. Determination of Exchangeable Potassium

Potassium was extracted from the soil by mixing 10 milliliters of 1 normal, neutral, ammonium acetate with 1 gram scoop of soil and shaken for 5 minutes. The exchangeable potassium was on emission mode at 776 mm. The results were reported as parts per million (ppm) of potassium (K) in the soil. Soil sample of 20 gm was well shaken with 40 ml of distilled water in 250 ml conical flask for an hour. The conductivity of the supernatant (saturation extract of soil) liquid was determined with the help of conductivity meter [40].

8. CONCLUSION

Endophytic microbes are a group of organisms living inside the host cell and exhibit symbiotic relationship with the host. The host plants are diverse and hence ubiquitous in nature. Endophytes are well known for their production of various plant growth promoting substances, protecting from environmental conditions, providing sustainability and an important nature of endophytes are they could produce a substance that acts with a flavor which will stop grazing the plant by an herbivore animal. Hence the present review focused on endophytic bacteria and various carrier material (ranging from bagasse to sawdust) to produce a carrier based inoculant which could be more helpful for the plant to withstand during adverse conditions.

Conflict of Interest

The authors of this manuscript do not have any conflict of interest towards this article publication.

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