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INFLUENC OF VARIOUS BACTERIAL CARRIERS ON SURVIVAL OF CERTAIN DIAZOTROPHS

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ABSTRACT: *A laboratory incubation experiment was performed to declare the efficiency of some materials (presterilized and sterilized) to serve as bacterial carriers for the diazotrophs Bradyrhizobium sp. (symbiotic) and Azospirillum sp. (associative). The carrier materials used were vermiculite 90% + peat 10% as "standard", compost, biogas manure and filter mud. The inoculated bacterial carriers were kept at 25 ˚C for a period continued up to 90 days. Survival of the bacterial inoculants was estimated, at intervals of storage, using specific culture media.*
The results denoted that the maximum bacterial growth rate, for both diazotrophs, occurred at the first interval, i.e. 7 days. Azospirillum greatly surpassed the other Bradyrhizobium in regard to the growth rate and viability with all carriers tested, all over the experimental duration. Order obtained for the bacterial carrier materials that suited the survival of the examined bacterial strains was: compost > biogas manure > standard (vermiculite + peat) > filter mud for both organisms. Generally, presterlization of the carrier materials favored the viability of the inoculating organisms, compared to the non-sterilized carriers.

Key words: *Bradyrhizobium, Azospirillum, compost, biogas manure, filter mud, vermiculite.*

INTRODUCTION

Biological nitrogen fixation "BNF" (diazotrophy) is carried out by various microbial agents (diazotrophs) mainly bacteria. These microorganisms are classified as free-living, associative and symbiotic. In order to augment the capacity of BNF in soil, it is usefully recommended to supply the soil with the specific diazotrophs. N_2-fixers are introduced to the soil in the form of inoculated carriers. A variety of materials have been used to carry the bacterial inocula (Yardin *et al.,* 2000; Rebah *et. al.,* 2007and Arara *et al.,* 2014). Thus, selection of certain carrier depends on its properties, which in turn favor the maintenance and viability of bacterial inoculants (Stephens and Rask, 2000).

Inoculants are commercially available as solid products, powder produced from peat, or as granular form, or liquid using broth medium (Stephens and Rask, 2000 and Rebah *et al.,* 2007). Carbon source (mannitol), nitrogen source (yeast extract), and mineral salts are used, which are expensive at a large or industrial scale (Rebah *et al.,* 2007). In many countries, the local development of commercial rhizobial inoculants is limited by technological limitations or the scarcity of local sources of peat. Currently, production of soybean inoculants in several developing countries including Egypt is limited, and hence they should be imported.

The development of locally produced inoculants is thus desirable, due to adaptation to the dominant conditions. To do so, it is necessary to find out carriers and preparation methods that are widely available and accessible locally. A suitable rhizobial carrier should have a suitable water holding capacity and a good aeration characteristic, to support the bacterial growth and survival, as well as to be non-toxic, easily sterilized, practically handled in the field, environmentally friend, and has a good storage quality. In addition, it is important for its production and utilisation, to be mixable, packageable, probably adhered to seeds, available as powder or granule, easily release inocula to the soil, and inexpensive (Bashan 1998 and Rebah *et al.,* 2002).

Whereas, three general categories of alternative materials are used: (a) soil materials including coal or mixtures of coal and other materials, mineral soils and

mixtures of mineral soils and other materials; (b) plant materials including bagas, rice husk, and other plant composts; and (c) neutral ingredients including vermiculite, mixtures of vermiculite and other materials, perlite, rock phosphate, calcium sulphate, and some polyacrylic gel synthetics (Rebah *et al.,* 2007 and Smith, 1992).

Development of a successful inoculant involves the selection of a suitable carrier substrate in order to support the growth of the target organism and maintain a high number of inoculanting bacteria. An appropriate carrier should contain some organic matter and optimum nitrogen content. Incorporation of microorganisms in a carrier material enables easy-handling, long-term storage and high effectiveness of biofertilizers. Although peat is the carrier of choice, it is not ubiquitously distributed, and thus a more readily available substrate may be required. Internative carriers have been investigated, including various clays, animal manure such as poultry manure, composted plant materials or other complex organic matrices (Stephens and Rask, 2000).

Therefore, a laboratory experiment was performed to examine a number of materials (either sterilized or non-sterilized), for their potentiality to serve as bacterial carriers for each of the diazotrophs *Bradyrhizobium sp*. (symbiotic) and *Azospirillum liboferum* (associative). Specific culture media were employed to determine the viability of the carried bacterial cells, at intervals of storing the inoculated carriers.

MATERIALS AND METHODS

This study was carried out in the laboratory, in order to evaluate the effect of some materials, to be used as bacterial carriers, on the viability of certain diazotrophic bacteria along three-month storage period (shelf time).

1. MATERIALS

1.1. Bacterial carriers tested:

Four different materials were used as bacterial carriers, *i.e.* vermiculite 90% + peat 10% (standard), compost, biogas manure and filter mud. Some properties of these materials were determined, following the methods of Cottenie *et al.* (1982) and data appear in Table (1).

Table (1): Characteristics of the used carriers.

Characteristics		Unit	Standard*	Compost	Biogas manure	Filter mud
Moisture content		%	2.45	21.00	22.6	23.3
Organic carbon			5.81	21.95	30.31	36.21
Organic matter			9.99	37.75	52.13	62.28
Water Holding Capacity (WHC)			201	265	321	428
Total macro-nutrients	N	%	0.10	1.52	1.75	1.01
	P		0.21	0.85	0.96	0.66
	K		0.45	1.30	1.44	0.72
Available macro-nutrients	N	mg kg^{-1}	20	273	365	235
	P		45	266	182	298
	K		70	273	175	300
pH (1:5,soil/water sus.)			6.98	7.24	6.74	6.14
EC (1:5,soil/water extr.)		dS m^{-1}	1.13	3.55	1.27	3.22

*Standard: vermiculite 90% + peat 10%.

1.2. Diazotrophs used

Strains of two bacterial dinitrogen fixers, namely *Bradyrhizobium* sp . *"B"* (symbiotic) and *Azospirillum liboferum* CRT1 *"A"* (associative) (kindly supported from the Depth . of Agric . Microbiol .,ARC.) were carried on the above listed materials.

Methods

A portion of the used carriers was priorly sterilized by means of gamma irradiation, and the rest was left unsterilized. For sterilization, three carton boxes (diminsions : 55 X 44 X 16 cm), each containing 200 g of each carrier (at average moisture content 20%) in polyethylene bags, were set up for gamma irradiation. Radiation doses (>50 kGy) were extrapolated using an equation provided by ANSTO*. Sterility of the carriers was confirmed by plating several dilutions of buffer suspensions of the irradiated material on appropriate medium and making growth observations.

At each storge internal (7,15,25,40,55,70 and 90) sample of the *Bradyrhizobium* carrier (2.3×10^9 cell g^{-1}) inoculated a yeast mannitol broth "YMB" (Vincent, 1970). Whereas, equal sample of that *Azospirillum liboferum* CRT1 inoculated a modified nitrogen-free (Nfb) agar (Nelson and Knowles, 1978), containing 0.2 g l^{-1} ammonium chloride and Congo red. Compositions of both media (per liter) were the following:

"YMB" medium: K_2HPO_4, 0.5g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; yeast extract, 1 g and mannitol, 10 g.

"Nfb" medium: sodium malate (5 g/l) as carbon source; K_2HPO_4 0.6 g; KH_2PO_4 6.0 g; $MgSO_4.7H_2O$ 0.8 g; NaCl 0.4 g; $CaCl_2$ $.2H_2O$ 0.1 g; trace element solution ($MnCl_4.$ $4H_2O$ 28 mg;$MnCl_2$ .4 H_2O 28 mg; $Na_4MoO_4.2H_2O$ 8 mg; $FeSO_4.7H_2O$ 631 mg;) EDTA 592 mg, biotin 50 mg, volume was completed to 1000 ml, pH 7.2, and 1.8 g agar was added to make up the semisolid medium. The media was supplemented with NH^4Cl (1 g l^{-1}), Congo red (25 mg l^{-1}) and cycloheximide (40 mg l^{-1}).

After 5-day inocubation at 25°C, of the inoculted culture media, bacterial counts were performed to ascertain the effect of storage duration on survival of the used diazotrophs carried on the chosen carriers.

RESULTS AND DISCUSSION

Data presented in Table (2) show the growth rates, on the specific culture media,of both diazotrophic bacteria *Bradyrhizobium* "*B*" and *Azospirillum* "*A*", carried on the various carrier materials, at intervals of storage time. Results revealed that, the greatest bacterial numbers appeared at the first 7-day detection period, while prolonging the storage period decreased such counts. Growth rate of "*A*" greatly excelled that of "*B*" at all estimation intervals and with all carriers employed, taking in considration the starting equal numbers of the inoculating bacterial cells and carrier sample.

The results generally indicated that, the inoculated compost carrier exhibited the highest bacterial population and followed desindingly by biogas manure, standard and finally the filter mud. Presterlization of the examined carriers led to increase the counts of the cultured bacterial strains, as compared with the non-sterilized counterparts.

The above-mentioned results pointed out that, despite the initially equal number of carried bacterial cells of both inoculating strains and cultured carrier sample, type of the carrier seemed to determine the growth rate of both the dinitrogen symbiotic "*B*" and associative "*A*" fixers.

This is actually referred to the type and composition of the carrier itself, where compost and biogas manure seemed to be favorable bacterial carriers, because of their rich nutritinal status (Table 1). The inferiority of filter mud, as a sugar industry waste from sugar cane, could be due its contents of

* ANSTO: Austrlian Nuclear Science and Technology Organization - ANSTO is using nuclear science to benefit industry, people and the environment. http://www.ansto.gov.au/

TABLE 2

hardly decomposable organic compounds such as lignin, phenols and waxes. Despit the availabity of vermiculite, and absence of peat locally, usage of such carrier is not rather preferred. Number of colonies reflected the higher growth rate of "*A*" than that of "*B*", due to the nature of each microorganism, i.e. endurance to storage conditions and specific rate of reproduction. Such findings support the conclusions of yardin *et al .,* 2000 ; Xavier *et al .,* 2004 and Albareda *et al.,* 2008.

Presterilization of the carrier materials favored the concerned bacterial strains, being referred to destruction of competing or antagonestic organisms in the medium (Khavazi *et al*., 2007). Both studied diazotrophs showed their peaks of growth rate and viability at the first incubation interval, i.e. after 7-days, and started to decline thenceafter. This went along with the standard growth curve of bacteria (Poole, 2013).

Kalra *et al.* (2008) reported that maximum viable population of rhizobia could be recovered in granular vermicompost followed by charcoal and FYM after six months of incubation. Raja Sekar and Karmegam (2009) concluded that though the number of viable cells decreased towards the subsequent months, the carrier material mix with higher proportion of vermicasts (3:1, 4:1, 5:1, 6:1 and 1:0) sustained more than 1 x 10^7 g^{-1} cells, which was the optimum viable range for field application, indicating that the carrier materials with high proportions of vermicasts and vermicasts alone are able to support the survival of *A. brasilense*. Raja Sekar and Karmegam (2010) reported that, the vermicasts of *E. eugeniae* supports the survival of more than 1 x 107 g^{-1} viable cells of *A. chroococcum*, *B. megaterium* and *R. leguminosarum* till the end of 10th month, which is longer than observed in lignite (4–5 months). Hence, vermicast alone or in high proportion with lignite or suitable materials could be used as carrier material for the longtime survivability of the biofertilizers.

Several reports (Skipper *et al.*, 1980; Olsen *et al.,* 1995 and Bashan 1998) suggested that a substantial proportion of the inoculant produced using nonsterile carrier was unsatisfactory for farmer use because of low populations of rhizobia and/or high numbers of contaminants.

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تأثير حوامل بكتيرية مختلفة علي مدي حيوية مثبتات معينة للنيتروجين الجوي

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الملخص العربي

أجريت تجربة معملية لاختبار كفاءة بعض المواد لاستخدامها كحوامل بكتيرية لكل من بكتريا "بريدي ريزوبيوم" (مثبت نيتروجيني تكافلي) و " ازوسبيريللم" (مثبت نيتروجيني مشارك المعيشة). وكانت هذه المواد هي حامل قياسي (٩٠% فيرموكيوليت + ١٠% ثرب"بيت")، كومبوست، سماد بيوجاز و طين مرشحات. وقد سبق تعقيم جزء منها (بأشعة جاما) والجزء الآخر ترك بدون تعقيم. وتم حفظ الحوامل سواء المعقمة وغير المعقمة بعد تلقيحها بالبكتيريا المعينة علي درجة حرارة ٢٥ م° لمدة استمرت حتي ٩٠ يوما. وجري قياس حيوية مثبتي النيتروجين المعنيين علي حواملها علي فترات من التخزين، وذلك بتنمية كل منهما علي بيئة متخصصة.

وأظهرت النتائج أعلي معدل نمو للميكروبين عند أول فترة تخزين (٧ أيام) ليبدأ في التناقص تدريجيا بعد ذلك حتي نهاية المدة (٩٠ يوما). وتفوق " الأزوسبيريللم" في حيويته عن "البريدي ريزوبيوم" في جميع متغيرات التجربة (الحوامل وفترات التخزين). وكان ترتيب الحوامل البكتيرية موحدا لكلا الميكروبين من حيث حيويتها علي مدي زمن التجربة هو: الكمبوست > سماد البيوجاز > الحامل القياسي > طين المرشحات. وأدي التعقيم المبدئي لمواد الحوامل إلي ارتفاع معدلات نمو وحيوية الميكروبين بصفة عامة عن تلك الحوامل التي لم يسبق تعقيمها.

Table (2): Log numbers of diazotrophic bacterial cells of *Bradyrhizobium* "*B*" and *Azospirillum* "*A*", at varying storage periods of the inoculated carriers.

Bacterial Carriers examined	Incubation period (days)													
	7		15		25		40		55		70		90	
	Diazotrophic bacteria													
	"*B*"	"*A*"	"*B*"	"*A*"	"*B*"	"*A*"	"*B*"	"*A*"	"*B*"	"*A*"	"*B*"	"*A*"	"*B*"	"*A*"
Sterilized carriers														
Standard*	8.71	9.13	8.46	8.47	8.16	8.08	7.21	7.80	7.14	7.71	7.02	6.88	6.89	6.49
Compost	9.79	10.72	9.50	10.41	9.23	9.72	8.72	9.73	8.49	9.72	8.17	8.17	7.93	7.12
Biogas manure	9.20	10.04	8.93	9.75	8.65	8.92	8.10	8.38	7.98	8.38	7.61	7.49	7.39	6.49
Filter mud	7.76	8.48	7.80	8.23	7.62	7.56	6.71	6.98	6.73	6.75	5.88	6.07	5.94	5.71
Non sterilized carriers														
Standard*	7.27	8.53	7.26	8.73	6.80	7.97	6.46	7.48	6.14	6.95	5.88	6.73	5.31	4.73
Compost	7.88	9.35	7.65	9.08	7.74	8.62	6.91	8.52	6.78	8.16	7.05	7.49	6.31	7.22
Biogas manure	7.27	8.99	7.46	8.80	7.26	8.06	6.67	7.76	6.60	7.34	6.25	6.91	6.00	5.47
Filter mud	8.41	8.22	6.44	7.97	6.03	7.70	5.68	6.46	5.47	6.44	4.93	4.76	4.41	4.40

*Standard carrier: vermiculite 90% + peat 10%.

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