Increasing shelf life of rhizobacteria formula with alginate in encapsulation during storage

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Abstract. Rhizobacteria are biological agents reported to be able to increase plant growth and suppress plant diseases (Plant Growth Promoting Rhizobacteria). In general, the use of biological agents in various rhizobacterial formulas in the soil is still not effective. This is due to the decrease in population during storage. This research aims to obtain rhizobacterial formulations, therefore it requires a rhizobacterial formulation technique so that the population can be maintained during storage so that it remains effective in suppressing disease development and plant growth. This study was conducted Factorial (Rhizoplane and storage time) with three replications. This treatment consisted of thirty treatment combinations (ten rhizobacteria and three types of storage time). The results showed that the population density could be maintained until 2, 4 and 8 weeks of storage with the alginate carrier, namely 10⁶ CFU.gr⁻¹.

1. Introduction
Rhizobacteria are microorganisms that belong to a group of bacteria found in plant roots and as PGPR (Plant Growth Promotion Rhizobacteria) biological agents reported to be able to suppress the development of plant diseases and stimulate plant growth [1]. The use of rhizobacteria directly without formulation can reduce the population density because they are susceptible to variations stress from environmental factors such as fluctuations in soil pH, high soil temperature and salinity, as well as predation of microorganisms in the soil [2]. Population decline inhibits quorum sensing [3] thus inhibiting the formation of biofilms in root colonization [4], affecting root morphology [5] and nitrogen fixation [6], influences the availability of phosphorus in the soil [7], secretion of phytohormones, and gene expression associated with plant resistance to stress conditions [8]. In this condition, it is exacerbated by the formulation process which decreases the ability and viability of rhizobacteria in the field and during storage so that a rhizobacterial formulation is needed.

Formulation is the process of forming a formula (product) consisting of biomass controlling agents and compounds to increase the durability and effectiveness of the product [9]. Under certain conditions the rhizobacterial formula cannot be applied to plants. This is due to several reasons, including: (i) decreasing the number of effective populations in the formula during storage [10], (ii) environmental conditions that do not support rhizobacterial formulations during storage. (iii) Damage to the balancing or protective compounds of rhizobacteria in the formula, (iv) the formula is too long stored [11]. As a result, the objectives of the formulation to maintain viability and protect rhizobacteria in a suitable microclimate and prevent a certain time period could not be achieved [12]. The use of tapioca flour and peat as a carrier was reported to reduce the density of rhizobacteria population along with increasing storage time [13]. Peat formulation reduces cell viability after 6 months of storage [14].

The decline in rhizobacterial population causes a decrease in the effect of PGPR so that it is not effective when applied in suppressing disease progression and plant growth. Therefore, a carrier material is needed...
in a formulation technique that can not only be stored for a relatively longer time so that the effective population of rhizobacteria can be maintained but can also be tolerant during transfer and application and the absence of contamination. Utilization of peat as a solid carrier material in formulas is generally found but its stability varies so that it affects the quality of the formula and the occurrence of product contamination [15]. Formulations with liquid carriers are relatively easy to use and simple in formula production but are unable to protect against rhizobacteria resulting in a decrease during storage, transportation and during application to plants [16].

Bioencapsulation is a rhizobacterial formulation technique that aims to protect biological agents in the soil so that they can release rhizobacteria slowly and over a relatively long period of time. The formula can also be increased by adding substances or nutrients to increase the growth of rhizobacteria, increasing population density during formulation.

2. Material and method

2.1 Place and Time
This research was conducted in the Laboratory of Basic Sciences and Plant Protection, Department of Agroecotechnology, Faculty of Agriculture, University of Sultan Ageng Tirtayasa in May 2020 - November 2020.

2.2. Materials and Methods
The materials used in this study were: rhizoplan bacterial isolate, Nutrient Agar (NA) medium, 70% alcohol, distilled water, heat-resistant plastic bags, aluminum foil, tissue, plastic wrapping, plastic straps, polybags, filter paper, and label paper. The tools used are: glass petridish, microtube, test tube, beaker, measuring cup, erlenmeyer flask, laminar air flow cabinet, magnetic stirrer, autoclave, oven, hot plate, vortex, digital scale, schoot bottle, slide, micropipette, pipettes, test tube racks, stirring rods, spritus lamps, loop needles, syringes, incubation rooms, buckets, and stationery.

2.3. Research Design
This study used a completely randomized design. The treatment consisted of two factors, namely superior rhizobacteria (Rp. Han-1.2, Rp. Han-1.4, Rp. Han-4.1, Rp. Han-5.1, Rp. Han-5.2, Rp. Han-6.2, Rp. Han-7.1, Rp. Han-8.2, Rp. Han-9.1, Rp. Han-9.2) and storage times (2, 4 and 8 weeks) and control. Each treatment and control combination were repeated three times so that there were 90 experimental units. The data were analyzed using variance, if they were significantly different, then continued with Duncan's New Multiple Range Test (DNMRT) at the 5% level.

2.4. Rejuvenation and Rhizobacteria Formulation
Rhizoplan isolates were obtained from the Laboratory of Basic Science and Plant Protection, Department of Agroecotechnology, Faculty of Agriculture, University of Sultan Ageng Tirtayasa, Serang (collection of Julio Eiffelt Rossaffelt Rumbiak) stored in 0.90% NaCl solution in a microtube. These isolates were rhizoplan isolates capable of suppressing the development of plant diseases and promoting the growth of chili plants. Rhizoplan isolates were rejuvenated using strike method on NA medium, incubated 1 x 24 hours (Figure). The propagation of rhizoplan consists of two stages, namely the preculture rhizoplan suspension made by moving 1 rhizoplan colony into 25 mL of NB in an Erlenmeyer flask (vol. 250) incubated on a horizontal rotary shaker (150 rpm) 1 x 24 hours at room temperature. Furthermore, the Main culture rhizoplan suspension by moving 1 mL of preculture into 50 mL of NB in erlenmeyer (vol. 100 mL) and incubated in the same way. The density of rhizoplans were measured at 10^6 CFU / mL using spectrophotometer. The formulation technique was done on aseptic conditions in laminar air flow. Main culture rhizoplans were put into a sterile sodium alginate solution and homogenized using a rotary shaker (150 rev / minute) for 60 minutes, then dripped by injection into a sterile CaCl2 (1.5% w / w) solution to form beads and stored for 1-3 hours. The granules are filtered using sterile stencil paper and rinsed twice. The granules were put into the NB medium for 1x24 hours on a rotary shaker in order to increase the rhizoplan population in the formula. Then the formula is filtered and rinsed with sterile distilled water twice and dried on laminar air flow [17]. The formula was stored at room temperature (25° C) for 2, 4 and 8 weeks storage times.
2.5. Rhizobacterial Viability on Formula

The viability of rhizoplan isolates in the formula was by using a series dilution technique, namely 1 gr of alginate added by 9 mL of sterile distilled water and homogenized with vortex. Rhizoplan suspension is diluted to $10^{-6}$. Next, 0.1 mL of a $10^{-6}$ dilution was poured into NA medium, leveled with a spatula and incubated for 48 hours [18]. The number of colonies that appeared was counted with a colony counter. The rhizobacterial population density were calculated using formula:

$$JB = A \times C$$

Note:

- $JB$ = Population density (CFU/gr)
- $A$ = Number of colonies formed
- $C$ = Dilution factor

3. Results

3.1. Rhizobacterial Viability on Formula

Rhizoplan was formulated with alginate as alginate carrier, was able to maintain viability stored for 2, 4 and 8 weeks. All rhizoplan were able to withstand up to 8 weeks of storage at a fixed density of 106 CFU/g. The number of colonies of rhizoplan isolates that grew on NA media was 20-69 colonies at 2 weeks of storage, while at 4 weeks the colony ranged from 22-63 and at 8 weeks the colony count ranged from 22-75 colonies. The number of rhizoplan colonies with the highest alginate formula was 75 isolates of Han.Rp-4.1 with 4 weeks of storage, while the lowest number of colonies was Han.Rp-5.2 with 2 weeks of storage.

All rhizoplan isolates could survive the alginate formulation at storage for 2, 4 and 8 weeks with population densities ranging from 7.30 - 7.88 Log CFU,gr⁻¹ (Figure 2) with a colony number of 20-75 (Table 1). There were three isolates of rhizoplan with increased population density with increasing storage time, namely Rp. Han-5.2, Rp. Han-6.2 and Rp. Han-8.2. Rp. Han-5.2. Isolates were stable in maintaining population density of Rhizobacteria until 8 weeks of storage.
**Figure 2.** Viability of the rhizoplan formula in the alginate formula with storage time of 2, 4 and 8 weeks.

**Table 1.** Population density of rhizoplan in alginate with different storage period times

| Rhizoplan | Population density (CFU.gr⁻¹) | Number of Colonies | 2 Weeks | Population density (CFU.gr⁻¹) | Number of Colonies | 4 Weeks | Population density (CFU.gr⁻¹) | Number of Colonies | 8 Weeks |
|-----------|-------------------------------|--------------------|---------|-------------------------------|--------------------|---------|-------------------------------|--------------------|---------|
| Rp.Han-9.1| 69 x 10⁶ | 69 a               | 36 x 10⁶ | 36 bc | 75 x 10⁶ | 75 a               |
| Rp.Han-4.1| 58 x 10⁶ | 58 a               | 75 x 10⁶ | 75 a | 46 x 10⁶ | 46 cd |
| Rp.Han-7.1| 52 x 10⁶ | 52 a               | 55 x 10⁶ | 55 b | 41 x 10⁶ | 41 cd |
| Rp.Han-9.2| 51 x 10⁶ | 51 ab              | 63 x 10⁶ | 63 a | 61 x 10⁶ | 61 bc |
| Rp.Han-1.2| 39 x 10⁶ | 39 bc              | 28 x 10⁶ | 28 cd | 22 x 10⁶ | 22 e |
| Rp.Han-5.1| 33 x 10⁶ | 33 cd              | 35 x 10⁶ | 35 bc | 30 x 10⁶ | 30 de |
| Rp.Han-1.4| 32 x 10⁶ | 32 cd              | 22 x 10⁶ | 22 d | 25 x 10⁶ | 25 de |
| Rp.Han-8.2| 27 x 10⁶ | 27 cd              | 37 x 10⁶ | 37 bc | 39 x 10⁶ | 39 de |
| Rp.Han-6.2| 26 x 10⁶ | 26 d               | 34 x 10⁶ | 34 bc | 48 x 10⁶ | 48 cd |
| Rp.Han-5.2| 20 x 10⁶ | 20 e               | 20 x 10⁶ | 20 e | 62 x 10⁶ | 62 bc |

KK : 24.93  kk : 26.45  kk : 27.12
4. Rhizobacteria Viability in Formula

All isolates were able to maintain a population density of \(10^6\) CFU.gr\(^{-1}\) which was formulated using alginate with a storage duration of 2, 4 and 8 weeks. Although there were differences in the number of colonies that appeared in some isolates, there was no change in population density between the time of formulation and the different storage times. Rhizobacteria were formulated using alginites to protect rhizobacteria from physical effects and reduce cell mortality with prolonged storage [19]. *Azospirillum lipoferum* can survive storage at room temperature for one year with a population density of \(10^6\) CFU.gr\(^{-1}\) [20]. *Azospirillum brasiliense* Cd and *Pseudomonas fluorescens* 313 which were formulated with dry alginate with 14 years of storage, did not lose their ability to increase growth in wheat plants with viability of \(10^5-10^6\) CFU.gr\(^{-1}\) [21]. This shows that the use of alginate in the formulation is in accordance with the purpose of the formulation, namely to maintain the viability of rhizobacteria during storage and when introduced to the soil [12].

Alginate is also friendly to the environment because it is biodegradable and is capable of releasing rhizobacteria gradually and in a prolonged manner [22]. The advantages of using alginate as a carrier in formulas are controlling the release of rhizobacteria in the soil, protecting rhizobacteria from abiotic (environmental) and abiotic threats, reducing contamination during storage and transport processes [23] and can be stored for a long time [17].

References

[1] Lugtenberg B and Kamilova F 2009 Plant-growth-promoting rhizobacteria. *Annual review of microbiology* 63 pp.541-556.

[2] Wu Z, Guo L, Qin S, Li C 2012 Encapsulation of *R. planticola* Rs-2 from alginate-starch-bentonite and its controlled release and swelling behavior under simulated soil conditions. *J Ind Microbiol Biotechnol* 39:317–327.

[3] Waters C M and Bassler B L 2005 Quorum sensing: cell-to-cell communication in bacteria. *Annu. Rev. Cell Dev. Biol.*, 21 pp.319-346.

[4] Zuniga A, Donoso, R A, Ruiz D, Ruz G A and González B 2017 Quorum-sensing systems in the plant growth-promoting bacterium *Pseudomonas* *putida* exhibit cross-regulation and are involved in biofilm formation. *Molecular Plant-Microbe Interactions*, 30(7), pp.557-565.

[5] Ortiz-Castro R and López-Bucio J 2019 Phytostimulation and root architectural responses to quorum-sensing signals and related molecules from rhizobacteria. *Plant Science*, 284, pp.135-142.

[6] Romero M, Muro-Pastor A M and Otero A 2011 Quorum sensing N-acylhomoserine lactone signals affect nitrogen fixation in the cyanobacterium Anabaena sp. *PCC7120*. *FEMS microbiology letters*, 315(2), pp.101-108.

[7] Lucero CT, Lorda G S, Ludueña L M, Anzuay M S and Taurian T 2020 Motility and biofilm production involved in the interaction of phosphate solubilizing endophytic strains with peanut, maize and soybean plants. *Rhizosphere*, 15, p.100228.

[8] Khan N, Bano A, Ali S and Babar M A 2020 Crosstalk amongst phytohormones from planta and PGPR under biotic and abiotic stresses. *Plant Growth Regulation*, pp.1-15.

[9] Schisler D A, Slininger P J, Behle R W and Jackson M A 2004 Formulation of Bacillus spp. for biological control of plant diseases. *Phytopathology*, 94(11), pp.1267-1271.

[10] Cassidy M B, Lee H and Trevors J T 1996 Environmental applications of immobilized microbial cells: a review. *Journal of Industrial Microbiology*, 16(2), pp.79-101.

[11] Dutta S and Podile A R 2010 Plant growth promoting rhizobacteria (PGPR): the bugs to debug the root zone. *Critical reviews in microbiology*, 36(3), 232-244.

[12] Bashan Y, de-Bashan LE, Prabhu S R, and Hernandez JP 2014 Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). *Plant and soil*, 378(1-2), 1-33.

[13] Rumbiak J E R, Habazar T, Yanti Y 2018 Introduksi Formula Rizobakteria *Bacillus thuringiensis* pv. *toumanoffi* Pada Tanaman Kedelai Untuk Peningkatan Ketahanan Terhadap Penyakit Pastul Bakteri (*Xanthomonas axonopodis* pv *glycines*) di Lapangan. *Jurnal Agroekoteknologi* 10(1).

[14] Fallik E and Okon Y 1996 Inoculants of *Azospirillum brasilense*: biomass production, survival and growth promotion of *Setaria italic* and *Zea mays*. *Soil Biol Biochem* 28:123–126.
[15] Deaker R., Roughley R J and Kennedy I R 2004 Legume seed inoculation technology—a review. *Soil Biology and Biochemistry*, 36(8), pp.1275-1288.

[16] Bashan Y, Hernandez J P, Leyva L A and Bacilio 2002 Alginate microbeads as inoculant carriers for plant growth-promoting bacteria. *Biology and Fertility of Soils*, 35(5), pp.359-368.

[17] Trivedi P and Pandey A 2008 Recovery of plant growth-promoting rhizobacteria from sodium alginate beads after 3 years following storage at 4 C. *Journal of industrial microbiology & biotechnology*, 35(3), pp.205-209.

[18] Klement Z., Rudolph K and San, D C 1990 *Methods in Phytophatology* (Akademia Kiado: Budapest, Hungary)

[19] Young C C, Rekha P D, Lai W A and Arun A B 2006 Encapsulation of plant growth-promoting bacteria in alginate beads enriched with humic acid. *Biotechnology and bioengineering*, 95(1), pp.76-83.

[20] Trejo A, De-Bashan L E, Hartmann A, Hernandez J P, Rothballer M, Schmid M and Bashan Y 2012 Recycling waste debris of immobilized microalgae and plant growth-promoting bacteria from wastewater treatment as a resource to improve fertility of eroded desert soil. *Environmental and Experimental Botany*, 75, pp.65-73.

[21] Bashan Y and González L E 1999 Long-term survival of the plant-growth-promoting bacteria *Azospirillum brasilense* and *Pseudomonas fluorescens* in dry alginate inoculant. *Applied Microbiology and Biotechnology*, 51(2), pp.262-266.

[22] Kim I Y, Pusey P L, Zhao Y, Korban S S, Choi H and Kim K K 2012 Controlled release of *Pantoea agglomerans* E325 for biocontrol of fire blight disease of apple. *Journal of controlled release*, 161(1), pp.109-115.

[23] Schoebitz M, López M D and Roldán A 2013 Bioencapsulation of microbial inoculants for better soil–plant fertilization. A review. *Agronomy for sustainable development*, 33(4), pp.751-765.