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BIOENCAPSULATION OF BIOCONTROL AGENTS IN BIOPOLYMER GEL MATRIX FOR INCREASING ITS SURVIVAL STABILITY AND BIOLOGICAL CONTROL ACTIVITY

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ABSTRACT: ***The present study described the bioencapsulation technique, which was useful for protection of biocontrol agent cells inside the biopolymer matrixes at room temperature. Spore forming biocontrol agents i.e. Bacillus subtilis (Bs-2) and Trichoderma hamatum (Th-18) survived very well for two years period. However, Pseudomonas fluorescens (Pf-5) survived poorly during storage. All bioencapsulated biocontrol agents significantly reduced Root rot on bean and sunflower caused by Sclerotium rolfsii. Seed coating was effective than soil amendment. B. subtilis was the effective biocontrol agent applied as seed coating or soil amendment.***

Key words*: Biological control, Biopolymer gel matrix, bioencapsulation, B. subtilis, T. hamatum and P. fluorescens.*

INTRODUCTION:

Synthetic chemical fungicides have been used to control plant diseases for many years ago. Due to chemical hazardous, environmental pollution problems and resistant to chemicals (Lumsden and Vaughn, 1993 and Koch, 1999), biological control has become a very important alternative strategy for plant disease control to overcome the problems resulted in using chemical pesticides in agriculture. The most biocontrol agents used successfully were *Trichoderma* spp., *Pseudomonas* spp. and *Bacillus* spp. (Cook and Baker, 1983; Lewis and Papavizas, 1985; Weller, 1988 and Kumar, 1998).

Microorganisms and other biocontrol agents are not very resistant to harsh natural conditions, weak bioactivities and short shelf life compared to synthetic agrochemicals and very fragile to hostile conditions (Burges, 1998).

Researchers have tried to overcome some of the weak points of biocontrol agents through development of new formulation technology to compete with cheap chemical pesticides, also large scale production of biocontrol agents should be feasible at low cost (Lewis and Larkin, 1997).

The polysaccharides starch and cellulose used to encapsulate pesticides for controlled release. Biocontrol agents have been encapsulated in chemical polymer matrices such as polyvinyl pyrollidone and polyvinyl alcohol (Baker, *et al.,* 1987).

Lewis and Papavizas, 1985, encapsulated *Trichoderma* sp. in an alginate-wheat bran mixture, Fravel *et al.,* (1985) tried to encapsulate various

microorganisms in a similar way using an alginate clay matrix. Another interesting method for encapsulation of biocontrol agents is entrapment in a starch matrix (Dunkle and Shasha, 1988 and Shasha and Dunkle, 1989). Extruded granular formulation with biomass of *Trichoderma* sp. and *Gliocladium virens* was developed by Lewis and Larkin, 1997 using vermiculite with rice flour.

Pregelatinized starch flour has been used to encapsulate biocontrol fungi (Lewis *et al.,* 1995) and to encapsulate biocontrol fungi and bacteria (Amer and Rania El-Shennawy, 2004). Biocontrol agents were encapsulated in granular formulation using wheat flour along with kaolin (Connick, *et al.,* 1991, 1997 and 1998) and semolina along with talc powder (Amer and Rania El-Shennawy, 2005).

The bioencapsulation matrix serves as a protective coating and provides enough nutrients for reproduction and production of bioactive compounds by cells inside the bioencapsulation matrix. *Sclerotium rolfsii* is a soil-borne plant pathogen of almost unlimited host range including bean and sunflower (Punja, 1985).

The aim of this work was carried out to use a very cheap natural biopolymer, most abundantly available agricultural products to:

1-Encapsulate some of biocontrol agents in biogelmatrix.

2- Test the shelf life of the encapsulated biocontrol agents.

3- Assess their activity in biological control of root rot of beans and sunflower as models of soil borne pathogen.

MATERIALS AND METHODS

Biocontrol agents production and harvest:

The biocontrol agents used were, two bacterial isolates (*Pseudomonas fluerescens,* Pf-5 and *Bacillus subtilis,* BS-2) and one fungal isolate (*Trichoderma hamatum,* Th-18). All effective biocontrol agents were obtained from Department of Agricultural Botany Collection, Faculty of Agriculture, Minufiya University, Egypt. Cultures were maintained on king's B (KMB) medium for Pf-5, nutrient glucose agar (NGA) for BS-2 and potato dextrose agar (PDA) for Th-18.

The biomass of each biocontrol agent was prepared after inoculation of 2L of early mentioned broth medium for each, prepared in 500 ml flasks each contain 150 ml broth medium and incubated in 250-rpm shaker at 28°C for 72 hrs for Pf-5, and Bs-2 and 15 days for Th-18.

The biomass of Pf-5 or BS-2 collected by centrifugation at 5000- rpm for 10 min, and the supernatant discarded. The resulting pellet re-suspended in 100 ml of phosphate buffer as final volume for each biogel and stored in refrigerator until use.

The biomass of Th-18 collected by filtration through double layer of muscline cloths, homogenized and stored in refrigerator until use. The filtrate centrifuged at 500 rpm for 10 min; the resulting pellet combined to homogenized biomass.

Preparation of biogel formulations:

The encapsulation materials are one or more carbohydrate rich biopolymers and/or one or more protein-rich biopolymers, were boiled and macerated to make a sticky gel. The biogel was autoclaved, cooled to room temperature and mixed with biocontrol agent cells or spores. This biogel-biocontrol agent complex was dried and crushed into powder.

The carbohydrate-rich biopolymers include rice, wheat, barley, corn, millet, potato, sweet potato, Taro (Dasheen), by-products and biopolymers extracted from these products.

Protein-rich biopolymers include many different types of beans, nuts, peanuts, cotton seeds, oil seeds, powders and by-products such as soyflour, soyproteins, meals of these products, and proteins extracted from these products.

The biogel encapsulation matrix was prepared by mixing the followed materials: soybean powder 50 gm, dasheen peel (taro) 50 gm, rice powder 25 gm, durum wheat flour 25 gm, vermiculite 250 gm, whey 2 gm, shimmed milk 3 gm, glucose 2 gm, $CaCO_3$ 1gm, Fe $SO_4.7H_2O$ 50 mg, Mn $Cl_2.4H_2O$ 10 mg and corn oil 10 ml. The ingredients used in biogel formulation were selected based on availability, cast effective, functionality and almost are agricultural products.

All ingredients were mixed together in 1L of deionized water and boiled at 100°C for 1 hr with stirring to make a uniform biogel matrix, the mixture was autoclaved at 121°C for 30 min. After cooling to room temperature, the biomass of each biocontrol agent, around 3.5 x 10^{12} cfu/ml spores or cells, was mixed with the desired biogel matrix, spread in thin layer on aluminium foil inside a laminar flow hood and dried at room temperature for 2 days. The dried biopolymer gel matrixs spores or cells complex were ground to obtain powders containing about 7x10^9 cfu/gm theoretically. The biogel matrix of bioencapsulated biocontrol agents were incubated at 28°C for 3 days before storage at room temperature.

Shelf-life and Survival of bioencapsulated biocontrol agents:

To determine the population dynamics of bioencapsulated biocontrol agents in the formulations over time, samples were drown at: (1) after preparation directly (original), (2) after 3 days of incubation, and (3) at intervals (2, 6, 12, 18 and 24 months) of storage at room temperature. Dilutions were prepared and 0.1 ml aliquots were plated on specified medium of each biocontrol agents. The population was assessed as colony forming

units (cfu). The independent samples were analyzed with three repticates. This experiment was set as a completely randomized design.

Effect of bioencapsulated biocontrol agents on root rot of bean and sunflower:

The pathogen *Sclerotium rolfsii* was isolated from diseased plants grown in Minufiya Governorate. Cultures of the pathogen were maintained on PDA.

The pathogen inoculum:

The pathogen was grown on wheat bran-vermiculite (1:1 w/w) in a polyethylene bags for 10 days at 28°C. Ten grams of pathogen inoculum containing actively growing mycelium along with sclerotia (approx. 3 gms of sclerotia/ 10 gm of inoculum) was used for each pot. Three parts of field soil and one part of well decomposed farm yard manure (FYM) were mixed together and filled in plastic pots of 25 cm diameter.

Seed treatment:

Seeds of Bean (Giza-6) as winter season crop or sunflower (Sakha-53) as summer season crop were coated with a thin layer of 1% carboxy methyl cellulose (CMC) and mixed with the powder of each individual bioencapsulated biocontrol agent at 4 gm/kg seed.

Bean seeds were coated with the formulations of initial time (after incubation of bioencapsilated biocontrol agents for 3 days just before storage) and sunflower seeds were coated with the formulations after 6 months of storage.

Ten coated seeds were sown per pot; some seeds were dressed with the fungicide Rovral (Iprodione) at the rate of 3 gm/kg seeds and used for comparative analysis. Pots inoculated with pathogen only and sown with surface sterilized seeds served as control.

Soil treatment:

The bioencapsulated biocontrol agents were added individually to inoculated pots at the rate of 3 gm/pot before sowing. 10 surface sterilized seeds were sown per pot. Each treatment comprised four replicates. The percentage of plants exhibited root rot symptoms were recorded after 30, 60 and 90 days after sowing.

RESULTS AND DISCUSSION

The described bioencapsulation technique was useful for protection of biocontrol agent cells inside the biopolymer matrixes. The total cell number increased ten fold during 2 days and 100 fold during 3 days of incubation. Most spore forming biocontrol agents survived very well during the bioencapsulation and drying process at room temperature.

During storage at room temperature, spore forming biocontrol agents such as *B. subtilis* (BS-2) and *T. hamatum* (h-18) survived very well for two years period. However, non-spore forming *P. fluorescens* (Pf-5) survived poorly during storage.

Shelf-life of bioencapsulated biocontrol agents was assessed over a period of two years at room temperature. There was an increase in biocontrol agent population within biogel matrix after incubation for three days, Table (1) and Figure (1). During storage, from an initial population (after three days of incubation) of 9.46 $\log^{-1}$ cfu/gm of biogel matrix, the population of *P. fluorscens* decreased down to 4.26 $\log^{-1}$ cfu/gm after 6 months and to 1.7 $\log^{-1}$ cfu/gm after one year of storage. The survival percent was 79% and 18% after 2 months and 6 months of storage respectively compared to the initial population.

Table (1):Stability of bioencapsulated biocontrol agents at room temperature.

Bioencapsulated biocontrol agents	Survival (%) after					
	Initial time*	2 months	6 months	1 year	1.5 year	2 years
P. fluorescens Pf-5	100	79	45	18	8	1
B. subtilis Bs-2	100	100	96	94	90	88
T. hamatum Th-18	100	98	95	92	87	81
L. S. D. for biocontrol agents (A) = 6.22 Periods = 3.15 A x B = 4.31						

*** Syrvival (%) during storage was calculated compared to the initial population (log cfu after 3 days of incubation) as starting time of each bioencapsulated biocontrol agent.**

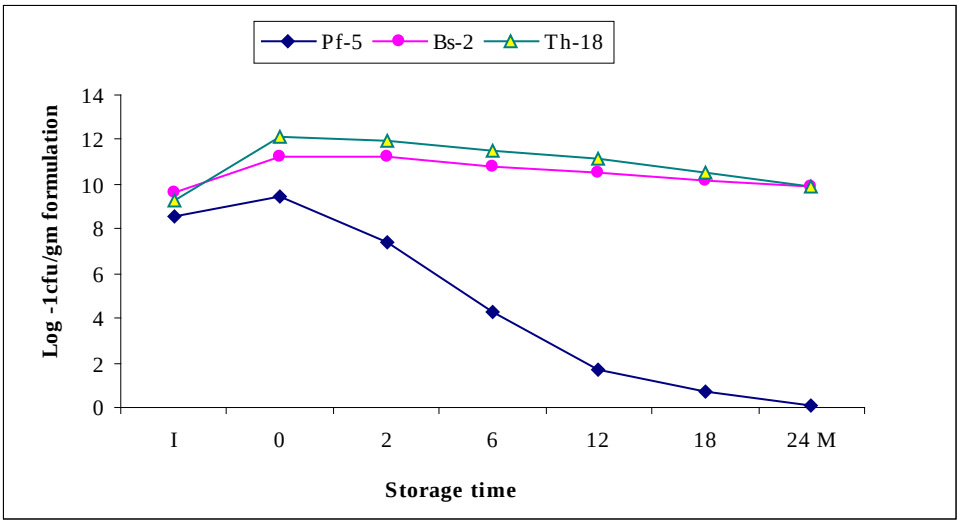

Fig. (1): Shelf life of bioencapsulated biocontrol agents.

For any commercial formulation the long shelf-life of the product is one of the pre-requisites. Vidhyasekaran and Muthamilan (1995) found that, *P. fluorescens* can survive well in talc or peat-based formulations for more than 8 months. *P. putida* population declined after 45 days of storage at different carriers (Amer and Utkhede, 2000). In pregelatinized corn flour powder formutation the survival of *P. fluorescens* was prolonged up to one year with 59% survival, also survived in semolina-tale powder up to 4 months of storage and declined after 6 months (Amer and Rania El-Shennawy, 2004 and 2005). *P. fluorescens* a non-spore former survived poorly during storage, it could be due to bioencapsulation and drying process.

***B. subtilis* shelf-life in the bioencapsulation biogel matrix was prolonged for 2 years of storage. The initial population was 11.26 $\log^{-1}$ cfu/gm of biogel matrix (Table 1 and Fig 1) and the population during storage period recorded 11.23, 10.83, 10.56, 10.16 and 9.9 $\log^{-1}$ cfu/gm after 2, 6, 12, 18 and 24 months respectively with survival percentages of 100, 97, 94, 90 and 84% for the same periods respectively.**

***B. subtilis* cells entrapped in biopolymer gel matrix seems to multiply in the biomatrix utilizing food sources present. *B. subtilis* can survive in certain dry formulations with effective populations (Turner and Backman, 1991). Also, suvived for 120 day in talc powder formulation, and up to one year in pregelatinized corn flour and semolina talc power formulations (Amer and Rania El-Shennays, 2003, 2004 and 2005).**

Shelf life of bioencapsulated *T. hamatum* in biopolymer gel matrix during storage for 2 years was initiated with 12.16 $\log^{-1}$ cfu/gm of biogel matrix (Table 1 and Fig 1) and the populations were 11.93, 11.53, 11.16, 10.56 and

9.86 log^{-1} cfu/gm during storage periods of 2, 6, 12, 18 and 24 months respectively. The survival percentages compared to the initial population were 98, 95, 92, 87 and 81% to the same periods of storage respectively. The biopolymer gel matrix formulation retained more than 81% of its initial population even after 2 years of storage. Lewis and Papavizas (1985) reported that isolates of *Trichoderma* spp. or *Gliocladium virens* intraped in alginate pellets or vermiculite/bran showed 60% viability up to 24 weeks at 25°C. *T. hamatum* survived for one year in pregelatinized corn flour with 92% survival of the original population, also survived in semolina-tale powder with 62% survival during storage at room temperature (Amer and Rania El-Shennawy, 2004 and 2005).

Effect of bioencapsulated biocontrol agents on root rot of bean and sunflower:

Generally, root rot on bean and sunflower plants caused by *S. rolfsii* was significantly reduced by all bioencapsulated biocontrol agents applied as seed coating or soil amendment as compared with the control. Seed coating was effective than soil amendment. Data presented in Table (2) show that, *T. hamatum* (T.18) was the most effective one in reducing root rot incidence percent on bean after 30 days of sowing when applied as seed coating or soil amendment recorded 4.76 and 4.8% disease incidence, respectively compared to control 62.66%. After 60 days of sowing, *P. fluorescens* was the most effective one and recorded 5.73 and 5.83%, disease incidence, respectively. After 90 days of sowing *B. sutilis* was the most effective one and recorded 0.83 and 1.30% of root rot incidence compared to control (4.1%).

The results with sunflower were in the same trend. The root rot incidence percent was higher than bean. *T. hamatum* the most effective biocontrol agent in reducing root rot incidence recorded 6.06 and 8.46% disease incidence, when applied as seed coating or soil amendment after 30 days of sowing dates if compared to control treatment (56.73%) and recorded 11.66 and 10.2% root rot incidence after 60 days of sowing, the soil amendment was effective than seed coating. After 90 days, *B. subtilis* was the effective biocontrol agent when applied as seed coating or soil amendment recorded 1.73 and 2.1% root rot incidence compared to control (9.23%). The fungicide Rovral was the most effective than other treatments after 30 and 60 days of sowing.

At the present time, there is considerable interest in the biological control of *S. rolfsii* through the introduction of biological control agents.

There are three strategies in considering biological control with introduced biocontrol agents: (a) to reduce the population of the pathogen; (b) to prevent the pathogen to infect the plant; and (c) to limit the disease development after infection (Cook, 1993).

Table (2): Effect of biogel encapsulated biocontrol agents on root rot incidence in bean and sunflower under potted condition.

Bioencapsulated bioagents /Application method	Root rot incidence % / days after sowing					
	Bean			sunflower		
	30 days	60 days	90 days	30 days	60 days	90 days
P. fluorescens Pf-5 (seed)	4.76	5.73	2.03	12.96	15.06	2.93
P. fluorescens Pf-5 (soil)	6.76	5.83	2.50	14.23	15.00	2.73
B. subtilis BS-2 (seed)	4.83	5.63	0.83	8.46	10.33	1.73
B. subtilis BS-2 (soil)	5.60	7.50	1.30	8.06	11.06	2.10
T. hamatum Th-18 (seed)	4.76	5.73	1.36	6.06	11.66	2.03
T. hamatum Th-18 (soil)	4.80	6.26	1.90	8.46	10.02	2.16
Fungicide (Rovral)	1.66	3.83	2.83	2.93	12.00	4.33
Control	62.66	13.66	4.10	56.73	23.70	9.23
L. S. D. for Biocontrol agents (A) = 3.16 Periods (B)= 2.03 A x B = 2.15				(A) = 4.61 (B)= 1.55 A x B = 3.46		

Seed coating with the bioencapsulated biocontrol agents reduced the incidence of root rots on bean and sunflower plants. This demonstrates the potential of seed coating to control the disease during the plant growth stages. This revealed that the biocontrol agents applied to seeds were able to grow along with germinating seeds and elongating roots, and protect the roots against infection with pathogens (Kloepper and Schroth, 1981).

The results obtained revealed that, soil application of bioencapsulated biocontrol agents was effective in reducing root rot incidence and this explained by the fact that biopolymer gel matrix in the soil acts as food base for biocontrol agents for period of time in the soil and lead to increase its

population, therefore the population of the pathogen was decreased. These results are correlated with the ability of biocontrol agents to parasitize the hyphe of *S. rolfsii* and antibiosis potential (Elad *et al.,* 1983; Henis *et al.,* 1984; Freitas and Pizzinato, 1997; Keel *et al.,* 1992).

REFERENCES

Amer G.A. and Rania Z. El-Shennawy (2003). Field application of powder formula of Bacillus subitillus for control damping-off and root rot of soybean. Minufiya J. Agric. Res., 28 (4): 1079-1091.

Amer G.A. and Rania Z. El-Shennawy (2004). Corn flour powder formula of antagonistic for biocontrol of chickpea seed rot and wilt diseases. Minufiya J. Agric. Res., 29 (4): 799-811.

Amer G.A. and Rania Z. El-Shennawy (2005). Efficiency of formulated biocontrol agents in control damping-off of root rot diseases of lupine (Lupinus termis) under field conditions. Minufiya J. Agric. Res., 30 (3): 853-866.

Amer, G.A. and R.S. Utkhede (2000). Development of formulations of biocontrol agents for management of root rot of lettuce and cucumber. Can. J. Microbiol., 46: 809-816.

Baker, C.A.; A.A. Brooks; R.Z. Greenley and J.M.S. Henis (1987). Encapsulation method. European patent 0320483.

Burges, H.D. (1998). Formulation of Microbial Biopesticides: Beneficial microorganisms, nematodes and seed treatments. Kluwer Academic Publishers, Netherlands, pp. 412.

Connick, W.J.; C.D. Jr. Boyette and McAlpine, J.R. (1991). Formulation of mycoherbicides using a pasta-like process. Biol. Control 1, 281-287.

Connick, W.J.; D.J. Daigle; A.B. Pepperman; K.P. Hebbar; R.D. Lumsden; T.W. Anderson and D.C. Sands (1998). Preparation of stable granular formulations containing Fusarium oxysporum pathogenic to narcotic plants. Biological control, 13: 79-84.

Connick, W.J. M.A. Jr. Hackson; K.S. Williams and C.D. Boyette (1997). Stability of microsclerotial inoculum of Colletotrichum truncatum encapsulated in wheat flour-kaolin granules. World J. Microbiol. Biotechnol., 13, 549-554.

Cook, R.J. (1993). Making great use of introduced microorganisms for biological control of plant pathogens. Ann. Rev. Phytopathol. 31: 53-80.

Cook, R.J. and K.F. Baker (1983). The nature and practice of biological control of plant pathogens. The American Phytopathological Society: St. Paul, Minnesota, U.S.A.

Dunkle R.L. and B.S. Shasha (1988). Starch-encapsulated Bacillus thuringiensis: a potential new method for increasing environmental stability of entomopathogens. Environ. Entomol. 17: 120-126.

Elad, Y.; I. Chet; P. Byle and Y. Henis (1983). Parasitism of Trichoderma spp. on Rhizoctonia solani and Sclerotium rolfsii, scaning electron microscopy and fluorescent microscopy. Phytopathology 73: 85-88.

Fravel, D.R.; J.J. Marois; R.D. Lumsden and W.J. Connick, Jr. (1985). Encapsulation of potential biocontrol agents in an alginate-clay mixture. Phytopathol. 75: 774-777.

Freitas, S.; S. Dos and M.A. Pizzinato (1997). Action of rhizobacteria on Colletotrichum gossypii incidence and growth promotion in cotton seedlings (Gosspium hirsutum). Summa Phytopathologica, 23: 36-41.

Henis, Y.; J.A. Lewis and G.C. Papavizas (1984). Interactions between Sclerotium rolfsii and Trichoderma spp. relationship between antagonism and disease control. Soil Biol. Biochem., 16: 391-395.

Keel, C.; U. Schneider; M. Mourhofer; C. Voisard; J. Laville; U. Burger; P. Wirthner; D. Haas and G. Defago (1992). Suppression of root disease by Pseudomonas fluorescens CHAO: importance of the secondary metabolite 2,4- diacetylphloroglucinol. Mole. Plant Microbe Interact. 5: 4-13.

Kloepper, J.W. and M.N. Schroth (1981). Relationship of in vitro antibiosis of plant growth-promoting rhizobacteria to plant growth and the displacement of root microflora. Phytopathology, 71: 1020-1024.

Koch, E. (1999). Evaluation of commercial products for microbial control of soil borne plant diseases. Crop Protection, 18 (2): 119-125.

Kumar, B.S.D. (1998). Disease suppression and crop improvement through fluorescent pseudomonads isolated from cultivated soils. World Journal of Microbiology and Biotechnology, 14: 735-741.

Lewis, J.A. and G.C. Papavizas (1985). Effect of mycelial preparation of Trichoderma and Gliocladium on population of Rhizoctonia solani and the incidence of damping-off. Phytopathology, 75: 812-817.

Lewis, J.A.; R.D. Lumsden; D.R. Fraval and B.S. Shasha (1995). Application of pregelatinized starch flour granule formulations of biocontrol fungi to control damping-off diseases caused by Rhizoctonia solani. Biol. Control 5: 397-404.

Lewis, J.A. and R.P. Larkin (1997). Extruded granular formulation with biomass of biocontrol Gliocladium virens and Trichoderma spp to reduce damping-off of eggplant caused by Rhizoctonia solani and saprophytic growth of the pathogen in soil-less mix. Biocontrol Sci. Tech. 7: 49-60.

Lumsden, R.D. and J.L. Vaughn (1993). Pest management: Biologically Based Technologies. American Chemical Society. Washington. DC.

Punja, Z.K. (1985). The biology, ecology and control of Sclerotium rolfsii. Annu. Rev. Phytopathol. 23: 97-127.

Shasha, B.S. and R.L. Dunkle (1989). Starch encapsulation of entomopathogens. U.S. patent, 4, 859, 377.

Turner, J.T. and P.A. Backman (1991). Factors relating to peanut yield increases after seed treatment with Bacillus subtilis. Plant Disease, 75: 347-353.

Vidhyasekaran, P. and M. Muthamilan (1995). Development of formulations of Pseudomonas fluorescens for control of chickpea wilt. Plant Dis., 79: 782-786.

Weller, D.M. (1988). Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annual Review of phytopathology, 26: 379-407.

تغليف كائنات التضاد اىوي ح ل فى جيل البوليمرات الطبيعيه لزيادة قدرتها على البقاء حيه ونشاطها فى المقاومة الحيوية

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الملخص العربى

هذه الدراسة توضح أهمية طريقة لي م ح ت ت ا ن ئ اك د اض ت ل ا اىوي ح ل على ماده جيلاتينيه فى شكل كبسولات فى حماية خلايا هذه الكائنات داخل هذه الكبسولات على درجة حرارة الغرفة. الجراثيم التى تكونها ت ا ن ئ اك د اض ت ل ا ىوي ح ل ا مثل باسيلس ستلس وتريكودرما هاماتم ظلت حيه وبحاله جيدة لمدة عامين بينما سيدوموناس فلورسنس كان بقائها حيه نادرا أثناء التخزين. قللت هذه الكائنات فى صورتها التى تم تحضيرها مرض عفن الجذور فى الفاصوليا وعباد الشمس المتسبب عن الفطر سكلورشيم رولفزياى بدرجه معنوية وكانت معاملة البذور أفضل من معاملة التربه. وكانت البكتريا باسيلس ستلس أحسن الكائنات كفاءة فى المقاومة سواء فى معاملة البذور أو معاملة التربة.