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INFLUENCE OF CHITOSAN SOLUTION ON THE VIABILITY OF ARBUSCULAR MYCORRHIZAL SPORES

Nalini Singh* Research Scholar NES Ratnam College Of Arts, Science & Commerce, Bhandup(West), Mumbai -78. *Corresponding Author

Sunita Chahar Head, Department of Botany NES Ratnam College Of Arts, Science & Commerce, Bhandup(West), Mumbai -78

ABSTRACT The study was aimed to fortify the Arbuscular Mycorrhizal Fungal (AMF) spores, widely used in Bio-fertilizers with chitosan and check the influence of chitosan on the viability of AMF spores. Chitosan was prepared from shrimp shells using chemical method involving demineralization, deproteinization and deacetylation.AMF spores were kept in three different concentrations of chitosan (0.1%, 0.5 %and 1%) which was prepared in 0.1% acetic acid and 0.1% ascorbic acid. Spore viability was checked by the MTT 3-(4, 5 dimethylthiazol-yl-2, 5-diphenyl-2H-tetrazolium bromide) after a day, 10th day, 20th day and 30th day. Highest number of viable spores was observed in chitosan dissolved in 0.1% ascorbic acid as compared to chitosan in acetic acid.

KEYWORDS : Arbuscular Mycorrhizal Fungi, Chitosan, Viability, MTT

INTRODUCTION

Arbuscular Mycorrhizal Fungi (AMF) are a group of root obligate biotrophs associated with about 80% of plants (5). They are considered natural fertilizers as they increase plants tolerance to biotic and abiotic stresses and induce disease resistance (4). In recent years, the AMF spores are cultured on roots in labs and are mixed with variety of carrier materials such as sand, vermiculite, clay, talcum powder, rice bran, charcoal, paddy straw compost, wheat bran, or a mixture of such materials, etc. which provide better shelf life to bio-fertilizer formulation. During manufacturing process and storage, viability of spores is affected, due to changes in pH, moisture content and temperature changes which is not analysed and it is of great concern. Chitosan is a carbohydrate derivate of chitin obtained from the shells of crustaceans. Both chitosan and chitin are composed of D-glucosamine and N-acetyl-D-glucosamine unit, which is linked by glycosidic bond β-(1→4). Chitosan has been a bio-fungicide, bio-bactericide, and bio-viricide, which shoots plant defence system against the pathogen, thus encouragingthe immune system of plants, fruits, and vegetables (19). As chitosan is a natural by-product it has less effect on human health as compared to chemical fertilizers. Therefore, the use of chitosan as a bio-organic fertilizer is considered as a good alternative to chemical fertilizer (14). Chitosan is easily soluble in any organic and inorganic solvent with pH less than 6 (7). Chitosan might maintain the viability and promote the activity ofsome beneficial micro-organisms such as mycorrhizal fungi and it stimulates the plant's natural defence system, leading to genetic, physiological and biochemical reactions (13).The aim of our study was to fortify AMF spores with chitosan to have synergistic effect. Therefore an attempt was made to prepare chitosan, add this chitosan to AMF spores by solubilizing it in organic solvents such as ascorbic acid and acetic acid and check the viability of spores upto 30 days of storage.

MATERIALS AND METHODS

Synthesis of Chitosan: Chitosan was produced by deacetylation of chitin which was extracted from shrimp shells (*Pandalus borealis*) by the procedure given by (12).

Experimental Design: Spores of *Glomus intraradices* were procured from TERI, New Delhi. The spores were kept in three different concentrations of chitosan solutions (0.1%, 0.5 %and 1%) which was prepared using two different solvents i.e 0.1% acetic acid and 0.1% ascorbic acid. The pH of the solutions was maintained 6.5 by using 0.1 N NaOH. The spores were allowed to remain in these solutions for a period of 30 days.

Viability Test: Spore viability was checked by the MTT 3-(4, 5 dimethylthiazol-yl-2, 5-diphenyl-2H-tetrazolium bromide) vital staining procedure (2, 3)**.** Spore suspensions were diluted 1:1 with a solution of 2 mg/mL MTT and incubated for 40 hrs. Using separate mini glass bottles, 25 spores per replicate were treated with MTT for 40 hours to allow maximum staining response (14). Treated spores were procured and observed under compound microscope.

RESULT

Synthesized Chitosan: Chitosan yield was 42%, colour of chitosan was off white with flaky texture**.**

Viability as observed by colour: After staining with MTT, the spores which turned their colour from yellow to bluish/ purplish were considered as viable spores and the spores which turned black or remained unstained were non-viable (10,14) .

Figure 1. Shows the comparison of viability of spores in chitosan dissolved in three different concentrations of acetic acid and ascorbic acid. The spores were isolated after a day and then at an interval of 10 days. The spores remained viable in all three concentrations upto ten days **(Fig 1: a-d, i-l, q-t)**, the inner contents of the spores turned bluish purple which implies that the spores were viable. Spores isolated after twenty days **(Fig 1: e, m, u)** and thirty days **(Fig 5: g, o, w)** showed decrease in viability in Chitosan solution prepared in acetic acid, the inner contents turned black in some spores and in some the wall turned black colour.

Spores in Chitosan solution prepared in ascorbic acid also showed black walls in 0.5% and 1% at the end of thirty days**(Fig 1 : p, x)**.The spores retained bluish purple colour in 0.1% ascorbic acid solution even after 30 days **(Fig 1: h)**

Viability as observed by Spore count: Table 1.Shows the comparative viable spore count up to 30 days in three different concentrations of chitosan prepared in two organic solvent i.e. acetic acid and ascorbic acid .

Twenty five viable spores were added to each replicate and there were three replicas for each concentration. Each replica contained 25 spores. Out of these 25 spores, viable spores ranged from 19.0± 2.6to 24.3± 0.5upto 10 days. Beyond this viability decreased in acetic acid solution compared to ascorbic acid solution. At the end of 30 days the spore viability in ascorbic acid was maximum ranging from 17.6± 0.5to 22.6± 2.0. In Acetic acid, spore viability after 30 days ranged from 10.3± 2.0to 13.6± 1.5.

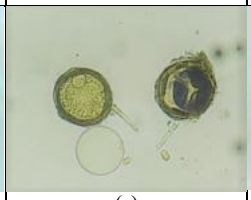
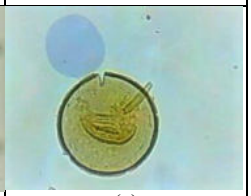
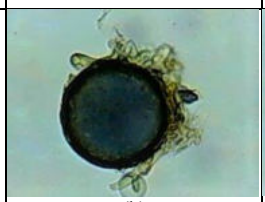
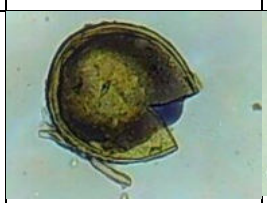
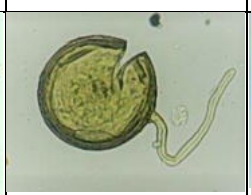
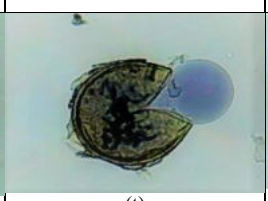
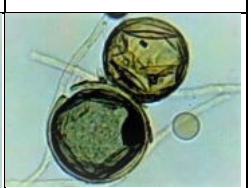
The maximum spore viability was observed in ascorbic acid solution ranging from 18.6± 1.2to 23.3± 1.5after a period of 20 days.

Table 1: Influence of Chitosan on the viability of AMF spores

Concentrations of chitosan		Viability (Day 1) No.of viable spores	Viability (Day 10) No.of viable spores	Viability (Day 20) No.of viable spores	Viability (Day 30) No. of viable spores
Chitosan (solution made in Ascorbic acid)	0.1%	24.3± 0.5	24± 1.0	23.3± 1.5	22.6± 2.0
	0.5%	23.0± 2.0	22± 2.0	20.3± 1.5	19.6± 0.5
	1.0%	21.3± 3.2	20.6± 0.5	18.6± 1.2	17.6± 0.5
Chitosan (solution made in Acetic acid)	0.1%	23.0± 1.0	20.6± 2.5	18.6± 1.5	13.6± 1.5
	0.5%	22.0± 1.7	19.3± 2.0	17.6± 1.6	12.3± 0.5
	1.0%	21.3± 3.0	19.0± 2.6	16.3± 1.1	10.3± 2.0

Mean value of three replicate with 25 spore per replicate ± Standard Deviation

Fig 1. Photographs of MTT Stained Spores Showing Viability.

Concentration Of Chitosan	Storage Period			
	1 Day	10 Days	20 Days	30 Days
0.1% Chitosan (dissolved in Acetic Acid)	 (a)	 (c)	 (e)	 (g)
0.1% Chitosan (dissolved in Ascorbic Acid)	 (b)	 (d)	 (f)	 (h)
0.5% Chitosan (dissolved in Acetic Acid)	 (i)	 (k)	 (m)	 (o)
0.5% Chitosan (dissolved in Ascorbic Acid)	 (j)	 (l)	 (n)	 (p)
1.0% Chitosan (dissolved in Acetic Acid)	 (q)	 (s)	 (u)	 (w)
1.0 % Chitosan (dissolved in Ascorbic Acid)	 (r)	 (t)	 (v)	 (x)

* All photos taken at 10 X magnification. ** Bluish purple contents in Spores - Viable *** Black contents/ Unstained- Non viable.

DISCUSSION

Viability of AMF spores is a very important aspect if they are to be used in Bio-fertilizers. The spores lose their viability when they are mixed in different carrier materials or during processing. Also the spores produced in- vitro are very delicate and have to be properly tested for their viability after they have been processed.

Although bio-fertilizers have been well established in the field of agricultural applications for many years, conventionally used solid and liquid formulations contain various problems associated with low viability of microorganisms during storage and application (6).

In the carrier-based bio- fertilizers, the microorganisms have a shelf life of only six months. They are not tolerant to UV rays and temperatures of more than 30 degrees. The count reduces day by day. But as compared to carrier based bio-fertilizer the shelf life of the microbes in liquid bio-

fertilizers is higher than carrier based bio fertilizers without considerable loss in viable counts and are also tolerant to high temperatures and UV rays. Liquid formulation application in the field is also very simple and easy (18). Liquid fertilizers are easy for use in drip irrigation as well as in hydroponics (6).

Chitosan is an organic material which is obtained from crustaceans, insects and fungi. It has numbers of commercial applications. In agriculture, chitosan is used as bio-pesticide, seed coating agent, as soil amendment and also enhances the post-harvest produce (9, 19). It was found that chitosan increases hairy root development (1). Chitosan also responsible for cytological alteration, protoplasm dissolution and large vesicle of fungus (15).

Chitosan dissolved well in acetic acid and ascorbic acids (7). Ascorbic acid is known to have growth regulating factor which influences many biological processes (11). It also improved the tensile strength and water uptake capacity of chitosan (7). Acetic acid was used as it can penetrate the soil surface and is easily decomposed by microorganisms and also has no possibility of biological accumulation or contamination (17). Both chitosan and Arbuscular mycorrhizal fungi together are beneficial for plant as it help plant to uptake necessary minerals and increases the productivity of plant (8).

We attempted to evaluate the viability of AMF spores in chitosan solution which could possibly be used in bio-fertilizer formulations along with other bio-inoculants.

CONCLUSION

Maximum viability in terms of spore count was achieved in 0.1 % chitosan dissolved in ascorbic acid. As both ascorbic acid and chitosan do not have any negative impact on natural environment, it can be concluded that chitosan maintains the viability of spores and can be used in the bio-fertilizer formulations.

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