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# Development of chitosan-PEG blended films using *Trichoderma*: Enhancement of antimicrobial activity and seed quality

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## 1. Introduction

Quality of the seed and inputs delivered through seed play a major role in productivity of crops as it is the basic unit in crop production. Even after many technological interventions in agriculture, most of the crops suffer losses due to poor quality of seed. Apart from the genetic and physiological purity of seed, biotic-abiotic stresses are significant deterrents in realizing optimum yields in crops. Employment of methods like seed treatment, soil amendment, foliar spray of antimicrobial agents, development of resistant varieties using gene modifications, good agricultural practices (GAP) etc. in general results in good plant stand and management of various stresses. Among the above described methods, seed treatment acts as a first line of defense against soil and seed borne pathogens for establishment of healthy crops [1,2]. Seed treatment refers to a process of application or coating seeds with physical, chemical and biological agents. Use of biological agents against harmful crop pests is described as biological control and now a days it is an emerging field due its eco-friendly nature.

Biological control is a process of controlling the pests of crops using beneficial microbial agents. Due to environmental and health hazards associated with chemical pesticides, biological control strategies are gaining importance. Among various beneficial microorganisms *Trichoderma* sp. are widely being used as biocontrol agent against several plant diseases. *Trichoderma* sp. like *Trichoderma harzianum* and *Trichoderma asperellum* are active rhizosphere colonizers [3, 4, 5, 6] and also known to colonize root cortical cells and exists as endophytes in crops [5, 7, 8]. Various secondary metabolites produced by *Trichoderma* are known to act as elicitors of defense response against biotic and abiotic stresses in crops. The antibiotics such as gliotoxin, viridin, cell wall degrading enzymes, and also biologically active heat-stable metabolites such as ethyl acetate produced by *Trichoderma* involve in disease suppression and/ or plant growth promotion. [9].

In agriculture, nowadays more emphasis is given on the use of biopolymers originated from plants or biological sources for seed coating which acts as seed stabilizing and protecting agents from biological and environmental stresses, enhance the nutrient absorption by plants resulting in plant growth promotion and yield improvement. A wide spectrum of natural polymers like polysaccharides, proteins, gums are available to use as suitable seed coating agents. Chitosan is one of the biopolymers employed in agricultural with wide application. Chitosan is a high molecular weight biological polysaccharide obtained by deacetylation of chitin, is a major

component of the shells of crustaceans (crabs, shrimps and crawfish) and cell walls of fungus [10]. Chitosan exhibited significant antimicrobial activity against yeasts, moulds, gram-positive bacteria and in a lesser extent against gram-negative bacteria [11]. Based on recent evidences, chitosan, when applied as a seed treatment, behaves as a resistance elicitor, inducing a physiologically enhanced defensive ability in seedlings and plants, potentiating the plant's innate defenses [12, 13, 14]. Combination of a biocontrol agent like *Trichoderma* along with other natural seed coating substance like chitosan polymer coats can enhance seed viability and germination under moisture stress and synergistic action against pathogen invasion. This type of delivery tactics involving polymer immobilization of *Trichoderma* can create a protective microenvironment around the seed, assuring adhesion of microbe to seed and may improve the plant growth.

Seed treatment with a combination of biocontrol agent like *Trichoderma* and chitosan-PEG blended system is a good strategy to protect seed from seed and soilborne pathogens and improves biocontrol efficacy [15]. The present study aims at development of chitosan based carrier system for delivery of *Trichoderma*. Because of its good adhesion and antimicrobial property, chitosan has been investigated as delivery carrier of *Trichoderma* for seed treatment. Likewise, polyethylene glycol (PEG) is a synthetic, biodegradable, water soluble polymer and acts an osmoprotectant for biocontrol agents like *Trichoderma*, so finds its application as chitosan-PEG (Cts-PEG) blended system for *Trichoderma*. In order to enhance the wettability on the plant surface and the adhesion of the blended system, incorporation of surfactants like glycerol to the blended matrices decreases the surface tension and maintains balance between the polar and non-polar groups. To optimize the concentrations of materials of blend, Cts-PEG-Gly blends prepared in different ratios of Cts, PEG and Gly was evaluated for seed quality attributes. The optimized chitosan carrier blend along with *Trichoderma* was studied for its physicochemical and biological parameters in order to widen its application under various conditions.

## 2. Material and methods:

### *2.1. Chemicals*

The chemicals for polymer synthesis like chitosan (Cts) (shrimp cells) with degree of deacetylation ≥ 75.0% as back bone of the polymer; polyethylene glycol 6000 (PEG); surfactants/ plasticizer like glycerol (Gly), glacial acetic acid (AAc) and other media for *Trichoderma* (Th) were acquired from Hi media Pvt, Ltd, Mumbai, India and further used without any purification.

*2.2. Biocontrol agent, Trichoderma spp.*

The fungal biocontrol agent *Trichoderma harzianum* (Th4d), which is a very potent strain having plant disease control and defense inducing capability, two other salt tolerant starins *T. asperellum* (A5) and *T. asperellum* (N13) with biological control potential were from *Trichoderma* culture collection maintained at IIOR, Hyderabad are used. These strains are fast growing at 25-30°C on potato dextrose agar (PDA) and turn green due to formation of conidia in a week.

The *Trichoderma* cultures were maintained on potato dextrose agar (PDA) slants and sub cultured once in every 30 days. For obtaining conidial powder, 2-3 agar culture disks of 5mm diameter in size from margins of actively growing *Trichoderma* culture were inoculated into the conical flasks of 250ml capacity containing about 150 ml of sterilized potato dextrose broth (PDB) and incubated in temperature controlled orbital shaker (M/s Scigenic Biotech Private Ltd, Chennai, India) for 3 days at 200 rpm and temperature maintained at 27 ±1 $^{0}$C. Then the culture broth with actively growing *Trichoderma* mycelium from the flasks was poured as thin layer into sterilized plastic trays (35 cm x 23 cm x 9 cm dimensions) with lids and left undisturbed for a four days. The green mat formed on the surface was carefully separated, dried in oven at 32 $^{0}$C, ground to fine powder using mortar-pestle and finely sieved to obtain conidial powder. The dry conidial powder so obtained was stored in high-density polyethylene (HDPE) bottles for further use.

*2.3. Synthesis of Cts-PEG and Cts-PEG-Th blended films*

Standardization of Cts-PEG blend using various concentrations of chitosan, PEG, glycerol was given in Table 1. Synthesis was carried out by slightly modified method as per earlier reports [16, 17]. The optimized Cts and PEG (1.5% w/v) was prepared by dissolving in distilled water using 0.1 % AAc till complete dispersion. Later, surfactant Gly (1% w/v) in the form of plasticizer was added to the above mixture. The reaction was allowed for overnight stirring at 60- 80 °C and 300 rpm. The blended system was allowed to induce gelatinization and Cts-PEG blended films were obtained for further studies.

After cooling of optimized Cts-PEG filmogenic solution, *Trichoderma* conidial spores was incorporated to a final concentration of 1% w/v ($10^{8}$ -$10^{10}$ CFUs). The blended dispersions were allowed on shaker for 15 min at 150 rpm to achieve a homogeneous distribution of spores. As a

control, a dispersion of *Trichoderma* spores in sterilized deionized water was prepared as per above mentioned concentrations [18].

*2.4. Seed treatment with developed filmogenic solutions*

Combinations of filmogenic dispersions prepared with different ratios of Cts, PEG and Gly were coated on seeds and evaluated for seed quality attributes like germination percentage and vigour index [19, 20]. Seeds of castor (*Ricinus communis* L.) variety DCS-107 were treated with Cts-PEG blend and untreated seeds served as control. For uniform seed coating, 100 g of castor seed was taken in a glass beaker along with polymer solution (20 ml) and shaken till the seeds coated uniformly. The coated seeds were air dried for 12-24 hrs at ambient room temperature. Precaution was taken during coating and drying of seeds to prevent clumping of seeds.

Coated seeds and uncoated seeds (as control) were studied for germination ability by paper towel method [21]. Twenty seeds were placed in moist paper towel in single row at equal distance and towels were rolled as bundle. Five replications were maintained for each treatment. The rolled paper towels were placed in a vertical position in a plastic tray and incubated in growth chamber at 27 ±1°C for 15 days. Proper moisture was maintained by adding sterilized distilled water in the tray as per requirement. Untreated seeds were kept as a check. The experiment was laid out in a completely randomized design. The data on percent germination, seedling growth (root and shoot length) and vigour index was calculated following Abdul-Baki and Anderson [20], on 16$^{th}$ day. Vigour index was calculated by multiplying germination percentage with the sum of root and shoot lengths.

*2.5. Effect of volume of filmogenic solution on germination and seedling vigour of castor*

In order to standardize the dose required to coat seeds with optimized film forming polymer (Cts-PEG-5), 100 seeds of castor for each replication were treated with different volumes of optimized film combination (Cts-PEG-5) and observations were recorded on germination (%), shoot length, root length and seedling vigour index by using paper towel method. The experiment was maintained in three replications.

*2.6. Compatibility of film forming materials with Trichoderma*

Compatibility tests were conducted under *in vitro* condition on *Trichoderma* growth (Th4d) using poisoned food technique [22]. The different concentration of materials are used for mixing in potato dextrose agar (PDA) for preparation of poisoned plates and *Trichoderma* culture discs of 5mm diameter from 5-day-old actively growing culture were placed in the middle of the agar plate. The plates without any amendment served as control. The plates were incubated at 25 ±1°C in an incubator. Percent inhibition of mycelial growth over the control was recorded 7 days after incubation as per formula mentioned below.

$$\text{Inhibition (\%)} = \frac{C - T}{C} \text{ X } 100$$

Where, C = Area covered by test organism in control (mm); T = Area covered by test organism in different treatments (mm)

*2.7. Effect of film forming materials on fungal pathogens*

The effect of polymer on the growth of the soil borne plant pathogens viz., *Sclerotium rolfsii*, *Fusarium oxysporum*, *Macrophomina phaseolina* and *Phytophtora nicotianae* was tested by poisoned food technique as mentioned above by adding chitosan at different concentrations *viz.*, 0.5, 1.0, and 2% in PDA. The percent inhibition of the pathogens was recorded on 8$^{th}$ day as per formula mentioned above.

*2.8. Effect of optimized Cts-PEG-5+ Th filmogenic solution on seed germination*

Effect of formulations under *in vitro* (paper towel method) and *in vivo* (pot experiment) was carried out for germination (%) and seedling vigour index of castor (*var.* DCS-107).

*2.9. Swelling*

The swelling property was determined by the slightly modified method of Pornpimon, et al., [23]. Swelling of a film is the measure of absorbed solvent until equilibrium when the film is immersed in any solvent. Initially, completely dried films ($W_i$) was immersed in 30 ml solvents and change in weights of films was monitored after 24 hrs of immersion. Solvents chosen for this study are acidic water, neutral water and alkaline water. Final weights of saturated films was taken ($W_f$) and swelling percentage was calculated using the following equation:

$$W\ (\%) = \frac{W_f - W_i}{W_i}\ X\ 100$$

*2.10. Characterization of developed films*

The film solution optimized Cts-PEG-5 was characterized for various physical and chemical characters after allowing the formation of films. A known volume of blend was poured in Petri plates and allowed to dry overnight to completely get rid of solvent (water) and films were obtained.

Characterization of Cts-PEG-5 films were done by Fourier-transform infrared spectroscopy (FTIR), X-ray powder diffraction (XRD), scanning electron microscopy (SEM) and thermal analysis. FTIR spectra of Cts-PEG films were recorded using Thermo Nicolet (is50) spectrometer using diamond ATR crystal. Scanning Electron Micrographs (SEM) were taken on JOEL-JSM 5600 at an accelerating voltage of 20 kV. X-ray Diffraction (XRD) studies were carried out using Bruker D8 X-ray powder diffractometer with Cu-Kα radiation (λ= 1.54 Å) source at voltage 40 kV and 30 mA current. Thermal analysis was done on thermogravimetric analysis (TGA) Q 500 (TQ instrument) through platinum pans. Small amount of film samples were scanned at a scan rate of 5 ºC/min on a Mettler-Toledo Differential scanning calorimeter (DSC) instrument. Data were recorded from -150 to 300 ºC in presence of nitrogen atmosphere.

*2.11. Spore germination assay*

Spore suspensions of *Trichoderma* from three months old chitosan liquid blended formulation (Cts-PEG-5+Th) (1 ml) was taken and prepared at a final spore concentration of $10^6$ to $10^{10}$ spores/mL by serial dilution in sterile distill water. The suspension of 100 µl from serial dilutions was placed in chambered cover glass devices (Lab-Tek II, Nunc; Thermo Fisher Scientific) for checking viability of *Trichoderma* in Cts-PEG-5 liquid blend stored at room temperature as per method described by Seiboth 2012 [24]. The slides with spore suspension were incubated for 12 h at 28 °C and checked for spore germination. The images of geminating spores of *Trichoderma* was taken at 40X zoom microscope (Nikon, Tokyo, Japan) using Nikon digital camera mounted on microscope.

*2.12. Statistical analysis*

The statistical analysis of the germination parameters was performed through an analysis of variance (ANOVA) using SPSS software 16.0 version. Critical differences were determined using CRD test (p =0.05).

## 3. Results and Discussion

### *3.1. Standardization of Cts-PEG films and effect on seed germination and seedling vigour*

Cts-PEG filmogenic blends were prepared with varying concentrations of chitosan, PEG and glycerol (Table 1). Among different combinations, Cts-PEG-5 (chitosan-1.5%, PEG-0.5% and Glycerol-1.0%) was selected as a choice of blend which has formed a uniform seed coating on castor seeds (*var.* DCS-107) which is an important factor for creating a perfect barrier on seeds against numerous seed and soil borne pathogens thus reducing the susceptibility to infections. Physical barrier formation on seeds due to seed coating helps in water absorption at an optimized rate in the vapour phase and protects the seeds in higher relative humidity [25].

Cts-PEG-5 blend coated castor seeds showed significantly higher seed germination (100%), root length (27.1 cm), shoot length (9.6 cm) and seedling vigour (3745) whereas chitosan treatment alone recorded low seed germination (75.0%) and seedling vigour (2170). Seeds treated with Cts-PEG-5 blend showed a significant increase in germination and vigour index compared to other combinations. Increased germination of castor seed treated with different blends over chitosan can be due to the presence of PEG (polymer) which imparts flexibility, stretchability and reduce brittleness facilitating proper coating of polymer. Glycerol (surfactant) imparts wetness to polymer assuring uniform adherence on the surface of the seed. Effective coating obtained with Cts-PEG-5 blend may be due to increased volume (1.0) of glycerol over other treatment results in increasing wetness of the composition and proper sticking on seed surface. Significant increase in seed germination with increasing volumes of glycerol can be observed (Table 1). Similar effect of plasticizers & surfactants have been observed in certain reports [26, 27].

### *3.2. Standardization of volume of filmogenic solution for seed coating*

In order to get a uniform seed coating and proper germination of seeds, volume of filmogenic solution should be optimized. Among different volumes used for castor seed coating, seeds coated with 10 ml of filmogenic solution showed significantly higher seed germination (93%) over other volumes of filmogenic solution and untreated control (Table 2). High seedling vigour index (4399) was observed with 10 ml filmogenic solution. Enhancement of germination in film coated seeds could be because of physical barrier on seeds created by the polymer which prevents seed from excessive moisture, dryness, freezing and physical damage while handling and

storage. Due to hydrocolloidal property of films, it provides ambient moisture required for germination of seeds resulting in better germination percentage. These findings were in corroboration with the earlier findings of Handiganoor et al., [28].

*3.3. Compatibility of ingredients of filmogenic solution with Trichoderma*

Chitosan did not show significant growth inhibition at all concentrations tested (Table 3a). PEG and glycerol @ 1% also did not inhibit *Trichoderma* growth significantly. As Trichoderma is compatible with the filmogenic reactants (Fig. 1 (i)), a combination of blended film with Trichoderma, a seed coating method can be developed. Compatible polymer provides congenial environment and facilitates maintaining the viability of the biocontrol agents [29]. Chitosan also stimulates the growth of Trichoderma compared to other pathogenic fungi. Chittenden & Singh, [30], González et al., [31] reported that the tolerance of *Trichoderma* with chitosan is associated with low membrane fluidity.

*3.4. Effect of ingredients of filmogenic blend on fungal plant pathogens*

Significant mycelial growth inhibition of four fungal pathogens was observed in different concentrations (0.5, 1.0, and 2.0%) of chitosan (Table 3b). Maximum inhibition of *Phytophthora nicotianae*, *Macrophomina phaseolina*, *Sclerotium rolfsii* and *Fusarium oxysporum* was seen @ 2% concentration of chitosan. More than 90% inhibition of mycelial growth of *Phytophthora* was observed in all three concentrations of chitosan. More than 50% inhibition of mycelial growth of Macrophomina, *Sclerotium* and *Fusarium* was recorded @ 1 and 2% concentrations of chitosan (Fig. 1 (ii)). The inhibition of pathogens due to chitosan indicates its anti-microbial activity [32].

*3.5. Effect of blended filmogenic -Trichoderma combination on seed germination and seedling vigour of Castor*

Chitosan filmogenic blend in combination with different isolates of *Trichoderma* was coated on castor seeds separately and germination in different treatments was recorded after 15 days. Optimized chitosan film blend alone (Cts-PEG-5), synthetic polymer, fungicide and a check without any seed treatment served as control. Significant increase in germination and vigour index was observed with all treatments over untreated control (Table 4). Highest vigour index (3110) was observed with *Trichoderma harzianum* (Th4d) treatment followed by chitosan film and *T. harzianum* (Th4d) combination formulation (3023) though the difference was non-significant.

Chitosan film alone treatment of castor seed also resulted in significantly higher vigour index (2975) compared to *T. asperellum* (A5) (2650), chitosan film + *T. asperellum* (A5) combination (2743) and *T. asperellum* (N13) (2680). Also, castor seed germination and seedling vigour in chitosan film coated seeds did not significantly differ from that of seeds coated with commercially available synthetic seed polymer indicating efficacy of chitosan polymer as a seed coating agent. *In vitro* and *in vivo* experiments also showed that seed coating with chitosan films did not hinder the germination of seeds and seedling vigour and in fact resulted in enhancement of seed germination and seedling vigour of castor. By priming of seeds with chitosan though not as blended polymer coat enhanced tolerance to chilling in maize by increasing seed germination speed [33]. Similarly, application of chitosan at optimum concentrations increased the germination and other studied growth factors [34]. Seed priming with chitosan improves saline tolerance in lentil plants irrespective of concentration of chitosan used [35]. Pretreatment of castor seeds with chitosan, biocontrol agent and fungicides enhanced the germination percentage and other growth parameters [36].

*3.6. Fourier-transform infrared spectroscopy (FTIR)*

Fig. 2, depicts the FTIR spectra of optimized Cts-PEG blend along with other reactants. Transmittance peaks in the 3300–3500 $cm^{-1}$ range were ascribed to the stretching vibration of OH groups. The variation in a 3280 $cm^{-1}$ in Cts-PEG blended film from 3289 $cm^{-1}$ in chitosan was attributed to the presence of glycerol. As mentioned before, glycerol as a surfactant agent has both polar and nonpolar fractions together and then its addition to the chitosan films could be result in binding with the polar fractions of the polymer such as hydroxyl groups and decrease the present O- H groups. A varied intensity band was observed at approximately 2950–2850 $cm^{-1}$ for all the reactants including chitosan film which could be attributed to the symmetric or asymmetric C − H vibrations or to the RNH and $RNH_2$ stretching vibrations. In addition, the peaks at 1375 $cm^{-1}$ and 1341 $cm^{-1}$, indicating that strong interactions occurred between chitosan and PEG components. Both chitosan and PEG possessed abundant OH and C-O groups, which could form hydrogen bonding and improve the compatibility between them. For PEG, the two peaks in the 1000–1400 $cm^{-1}$ region were ascribed to the C-O vibration of C-OH groups. For chitosan, the peak at 1025 $cm^{-1}$ was attributed to C-O vibration of C-O-C groups, and the bands at 1000 $cm^{-1}$ were ascribed to the C-O stretching vibration of C-OH groups.

*3.7. Thermal behavior*

*3.7.1. Differential scanning calorimetry (DSC)*

The DSC curve was examined to evaluate its characteristics of Cts-PEG film, which depend on aspects such as reaction and interaction between PEG and glycerol. Fig. 3A, shows the phase transitions observed. At very low temperatures (-144 °C) taken at midpoint, first endothermic event was observed which can be attributed to the glass transition temperature ($T_g$) of polymer. One exothermic effect (~ -134 °C) occurred above Tg, most likely due to the crystallization of species that do not crystallize during cooling. The melting transition was indicated by a broad endothermic event (46 to 62 °C) at 54 °C, which corresponded to the dissolution of crystallized fractions in the hydrocarbon matrix. The extent of crystallizable fractions is commonly associated with the presence of PEG in Cts-PEG blended system, which is responsible for the pavement exudation and inappropriate thermal susceptibility. The $T_g$ of plasticized films decreases with the increase in plasticizer contents, which is in agreement with the free volume theory of plasticization [37].

*3.7.2. Thermogravimetric analysis (TGA)*

Fig. 3B, represents the thermogravimetric curves of Cts -PEG blended film under inert atmosphere. The initial decomposition temperature ($T_{di}$) occurred at 250 °C. Under an inert atmosphere, two decomposition events were observed. The second event presented a decomposition temperature ($T_d$) of approximately 320 °C, near to 50% decomposition at 300 °C. At temperatures above the decomposition, there was a strong stability between the two events of decomposition. The complex structure of the Cts -PEG blended film was presenting a high reactivity between chitosan and PEG (polar groups) which is confirmed through FTIR [38].

*3.8. X-ray Powder Diffraction (XRD)*

X-ray diffraction allows the percentage of crystallinity observed for optimized Cts-PEG blended polymers. The diffractograms of Cts-PEG blended polymers, indicated two types of structural behavior (Fig. 3C). The structure of Cts-PEG differed from that of films of chitosan [39]. The addition of PEG and glycerol to film causes the loss of all crystalline structure in the membrane. The semi-crystalline to amorphous parts of each diffractogram were observed and the amorphous contribution is the one that confers the calculated baseline and the crystallinity results

from subtracting the baseline from the total diffractogram. The chitosan chain not only inhibits the crystallization of PEG, but also the hydrogen bond interaction between chitosan and PEG restrains the movement of chitosan molecules and even partially destroys the original crystalline structure of chitosan, and consequently decreases chitosan crystallization upon blending.

*3.9. Scanning electron microscope (SEM)*

Scanning electron microscope (SEM) microphotographs of the surface of Cts -PEG blended film (control) and Cts-PEG-Th blended film were shown in Fig. 4a and Fig. 4b respectively. In Cts-PEG polymer system displayed the rough textured surface, which is relatively flat in pure chitosan [40]. The rough texture on the surface of chitosan film is due to the reaction and plasticizing effect with the polar networks of PEG and glycerol. In Fig. 4b, showed that there are heterogeneous surfaces with undulation in Cts-PEG-Th blended film is due to the presence of spores of *Trichoderma* and smooth surface is due to chitinase activity of *Trichoderma*. Cts-PEG blended system is similar to the surface of chitosan–silica hybrid membranes [41].

*3.10. Swelling*

As shown in Fig. 5, swelling percentage of the optimized Cts-PEG films in various types of water plays an important role in agricultural application. The swelling percentage was maximum in neutral water (453), followed by acidic water (433) and there is a significant reduction in swelling percentage in alkaline water (180). In acidic water, swelling is slightly reduced, due to protonation of $–COO^-$ groups and elimination of repulsive forces. In alkaline water, the swelling reduction is due to charge screening effect [42].

*3.11. Spore germination assay*

Viability of *Trichoderma* in formulation is considered a determining factor for its application at field level in agriculture [43]. In the present study, 3 months old chitosan blended formulation was checked for *Trichoderma* spore viability by germination assay. Spore germination assay is a sensitive method in order to analyze the effect of any substance on fungal growth, development and inhibition [24]. In the present context, good germination of *Trichoderma* spores was observed in serial dilutions of $8^{th}$ to $10^{th}$ indicating its viability being maintained in blended formulation (Fig. 6). The addition of PEG and glycerol in formulation blend resulted in retaining the viability of spores during storage due to osmoprotectant nature of PEG and glycerol. As per the

quality control guidelines of CIB (Central Insecticide Board), Government of India which is a regulatory authority in India, the minimum viable counts/spores of 2 x $10^6$ per ml is required in any *Trichoderma* based formulations [44]. In the represent study for Cts-PEG+5 –Th blended formulation the viable spores being maintained at $10^8$-$10^{10}$ spores/ml of formulation which meets the requirement of CIB, India. The study on the shelf life and efficacy of Cts-PEG-5 with *Trichoderma* for one year is underway which will be further communicated in our future reports.

## 4. Conclusion

The Cts-PEG blended filmogenic solution with plasticizer can be used as a carrier for *Trichoderma* strains to be applied as seed coating material. The polymers provided uniform film formation after coating on any substrate and also on seeds, these coatings can modulate the exchange of gases and transmission of water vapor, without inhibiting seed germination and providing compatibility and protection during storage to *Trichoderma* spp. And rejuvenation of sporulation of *Trichoderma* under favorable conditions which is confirmed during seed germination. The coating appeared to be translucent and green color without and with *Trichoderma* respectively. SEM micrographs showed rough texture of Cts-PEG blend confirming film formation and entrapment of *Trichoderma* spores. The reaction was confirmed by structural characterization like FTIR, XRD, TGA and DSC. The developed materials must be further exploited for study of viability of *Trichoderma* in the films over a function of time and its potential in varied conditions of agricultural applications for its antimicrobial activity against plant pathogens other beneficial effects to crops.

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**Figure Captions:**

**Fig. 1. i.** Compatibility of ingredients of blended filmogenic solution with *Trichoderma*- a) Control, b) Chitosan 1.5%, c) 1% PEG, and d) 1% Glycerol; **ii.** Antimicrobial activity of chitosan against plant pathogens- a) *Sclerotium rolfsii*, b) *Phytophthora nicotianae*, c) *Macrophomina phaseolina*, and d) *Fusarium oxysporum*. (Concentrations of chitosan I) 1.5%, II) 2% , and III) 0% (Control))

**Fig. 2.** FTIR peaks of a) Cts-PEG film, b) Cts, c) PEG, and d) glycerol.

**Fig. 3.** Characterization of optimized Cts-PEG film A) DSC curve; B) TGA curve; C) XRD diffractogram

**Fig. 4.** SEM images of a) Cts-PEG film, and b) Cts-PEG-*Th* film

**Fig. 5.** Effect of water quality on swelling of Cts-PEG blended film at room temperature

**Fig. 6.** Microscopic images of spore germination of *Trichoderma* in 3 month old Cts-PEG-5 filmogenic blend at concentration of $10^8 – 10^{10}$ spores/ ml of formulation

**Abstract:**

Fungi under genus *Trichoderma* as ameliorates of biotic and abiotic stresses in cultivated crops is gaining popularity world-wide and their application in conjunction with seed coating polymers is an attractive proposition to reduce bioagent wastage and harnessing benefits of combined application. The synergistic action of *Trichoderma* with natural polymers like chitosan can enhance antimicrobial activity. A series of blended film solutions were synthesized by using chitosan, PEG and plasticizer in varying concentrations. The optimization of blended film composition and dose for coating of seeds was done w.r.t seed coating. Studies on compatibility of film forming ingredients with *Trichoderma* have not shown any inhibition and antimicrobial activity has shown different levels of inhibition of plant pathogens. Films were structurally characterized by XRD, SEM, FT-IR, TGA, DSC. The optimized film solution in combination with different *Trichoderma* strains improved seed quality parameters in test crop castor (*Ricinus communis*). Significant increase in vigour index (3110) was observed with Th4d treatment followed by chitosan and Th4d combination formulation (3023). In conclusion, the optimized chitosan-PEG-Th blend was effective in enhancing seed germination and plant growth of castor. The material can be further tested under large field evaluation as a seed coating agent against various plant diseases.

**Keywords:** Castor, chitosan, PEG, Glycerol, *Trichoderma*, seed quality, seed coating polymer.

**Table 1**
Standardization of blended filmogenic solutions and its effect on germination and vigour index in Castor *Ricinus communis* (*var.* DCS-107)

| Chitosan blend | Chitosan (%w/v) | PEG (%w/v) | Glycerol (%w/v) | Germination (%) | Shoot Length (cm) | Root Length (cm) | Vigour index |
|---|---|---|---|---|---|---|---|
| Cts | 0.5 | 0 | 0.1 | 75.0(60.0)$^{c}$ | 6.2$^{d}$ | 22.1$^{c}$ | 2170$^{d}$ |
| Cts -PEG-1 | 0.5 | 0.5 | 0.0 | 78.0(62.0)$^{c}$ | 6.2$^{d}$ | 22.2$^{c}$ | 2239$^{cd}$ |
| Cts -PEG-2 | 1.0 | 0.5 | 0.1 | 80.0(63.4)$^{c}$ | 8.2$^{b}$ | 19.3$^{d}$ | 2188$^{cd}$ |
| Cts -PEG-3 | 1.5 | 0.5 | 0.5 | 95.0(76.2)$^{ab}$ | 7.7$^{c}$ | 24.1$^{b}$ | 3003$^{b}$ |
| Cts -PEG-4 | 1.5 | 0.5 | 0.75 | 94.0(75.8)$^{b}$ | 5.9$^{d}$ | 19.5$^{d}$ | 2314$^{c}$ |
| Cts -PEG-5 | 1.5 | 0.5 | 1.0 | 100.0(89.0)$^{a}$ | 9.6$^{a}$ | 27.1$^{a}$ | 3745$^{a}$ |
| **SEm ±** | | | | 10.05 | 0.02 | 0.65 | 5475.11 |
| **C.D.(p=0.05)** | | | | 5.64 | 0.28 | 1.43 | 131.6 |
| **C.V(%)** | | | | 3.66 | 2.22 | 3.62 | 2.84 |

Values in parentheses indicate angular transformed values of germination percentage. When $p < 0.05$ ANOVA Tukey statistical test (95% confidence interval) was performed. Means within a column followed by different letter are significant at 5% level of significance and those following by the same letter do not differ significantly at 5% level of significance.

**Table 2**

Standardization of volume of blended filmogenic solution for seed coating in Castor (*var.*DCS-107)

| Volume of polymer/kg seed | Germination(%) | Shoot Length (cm) | Root Length (cm) | Vigour index |
|---|---|---|---|---|
| Control | 72.0(58.8)$^{d}$ | 15.9$^{c}$ | 27.9$^{c}$ | 3249$^{f}$ |
| 4 ml | 85.0(7.2)$^{c}$ | 16.3$^{b}$ | 28.3$^{c}$ | 3903$^{e}$ |
| 6 ml | 86.0(68.0)$^{c}$ | 16.6$^{b}$ | 31.2$^{a}$ | 4105$^{c}$ |
| 8 ml | 87.0(68.8)$^{c}$ | 17.4$^{a}$ | 30.4$^{b}$ | 4186$^{b}$ |
| 10 ml | 93.0(74.7)$^{a}$ | 17.1$^{a}$ | 30.3$^{b}$ | 4399$^{a}$ |
| 12ml | 90.0(71.5)$^{b}$ | 13.7$^{d}$ | 30.7$^{ab}$ | 3995$^{d}$ |
| **S.Em ±** | 2.22 | 0.05 | 0.14 | 408.5 |
| **C.D.(p=0.05)** | 2.65 | 0.42 | 0.67 | 35.9 |
| **C.V(%)** | 1.74 | 1.48 | 1.27 | 0.50 |

Values in parentheses indicate angular transformed values of germination percentage. When $p < 0.05$ ANOVA Tukey statistical test (95% confidence interval) was performed. Means within a column followed by different letter are significant at 5% level of significance and those following by the same letter do not differ significantly at 5% level of significance.

**Table 3a**

Compatibility of ingredients of blended filmogenic solution with *Trichoderma*

| Treatments | Mycelial Growth (cm) | Growth inhibition (%) |
|---|---|---|
| Cts (0.5%)+PDA | 4.5 | 10[c] |
| Cts (1%)+PDA | 4.2 | 16[b] |
| Cts (1.5%)+PDA | 4.1 | 18[ab] |
| Cts (2%)+PDA | 4.0 | 20[a] |
| Gly (1%)+PDA | 4.0 | 20[a] |
| PEG (1%)+PDA | 4.0 | 20[a] |
| Control | 5.0 | - |
| **S.Em ±** | 1.66 | |
| **CD (P=0.05)** | 2.29 | |
| **C.V(%)** | 7.74 | |

When $p < 0.05$ ANOVA Tukey statistical test (95% confidence interval) was performed. Means within a column followed by different letter are significant at 5% level of significance and those following by the same letter do not differ significantly at 5% level of significance.

**Table 3b**

Bioassay of ingredients of blended filmogenic solution on fungal plant pathogens

| Treatment | Growth inhibition (%) | | | |
|---|---|---|---|---|
| | ***Phytophthora nicotianae*** | ***Macrophomina phaseolina*** | ***Sclerotium rolfsii*** | ***Fusarium oxysporum*** |
| Cts (0.5%) | $89.5^{b}$ | $43.2^{c}$ | $50.2^{c}$ | $30.5^{c}$ |
| Cts (1%) | $100^{a}$ | $55.4^{b}$ | $58.6^{b}$ | $50.6^{b}$ |
| Cts (2%) | $100^{a}$ | $56.9^{a}$ | $65.9^{a}$ | $64.2^{a}$ |
| Gly (1%) | $1.2^{c}$ | $2.4^{d}$ | $2.7^{d}$ | $2.6^{d}$ |
| PEG (1%) | $0.2^{c}$ | $3.1^{d}$ | $2.7^{d}$ | $2.3^{e}$ |
| Control | - | - | - | - |
| **S.Em ±** | 0.51 | 0.68 | 0.26 | 0.02 |
| **C.D.(p=0.05)** | 1.38 | 1.5 | 0.94 | 0.28 |
| **C.V(%)** | 1.23 | 2.56 | 1.43 | 0.52 |

When $p < 0.05$ ANOVA Tukey statistical test (95% confidence interval) was performed. Means within a column followed by different letter are significant at 5% level of significance and those following by the same letter do not differ significantly at 5% level of significance.

**Table 4**

Effect of blended filmogenic solution- *Trichoderma* combination on seed germination and seedling vigor of Castor

| Treatments | Germination (%) | Shoot length (cm) | Root length (cm) | Vigour index |
|---|---|---|---|---|
| Cts-PEG | 96.6(78.9)$^{b}$ | 11.8$^{a}$ | 19.0$^{de}$ | 2975$^{c}$ |
| Th4d | 100.0(86.6)$^{a}$ | 10.0$^{c}$ | 21.1$^{a}$ | 3110$^{a}$ |
| Cts-PEG + Th4d | 96.6(78.9)$^{b}$ | 11.6$^{a}$ | 19.7$^{c}$ | 3023$^{b}$ |
| A5 | 100.0(86.6)$^{a}$ | 9.6$^{d}$ | 16.9$^{g}$ | 2650$^{i}$ |
| Cts-PEG + A5 | 96.6(78.9)$^{b}$ | 9.5$^{d}$ | 18.9$^{e}$ | 2743$^{f}$ |
| N13 | 100. (86.6)$^{a}$ | 9.1$^{ef}$ | 17.7$^{f}$ | 2680$^{h}$ |
| Cts-PEG + N13 | 93.3(74.8)$^{a}$ | 9.4$^{de}$ | 19.5$^{cd}$ | 2696$^{g}$ |
| Commercial seed polymer | 96.6(78.9)$^{b}$ | 11.1$^{b}$ | 19.0$^{de}$ | 2907$^{e}$ |
| Vitavax (fungicide) | 100.0(86.6)$^{a}$ | 8.93$^{f}$ | 20.5$^{b}$ | 2943$^{d}$ |
| Untreated Control | 86.6(68.2)$^{d}$ | 9.6$^{d}$ | 16.5$^{g}$ | 2260$^{j}$ |
| **S.Em ±** | 0.01 | 0.03 | 0.11 | 312.6 |
| **CD (P=0.05)** | 0.16 | 0.30 | 0.56 | 6.73 |
| **C.V(%)** | 0.09 | 1.80 | 1.75 | 0.14 |

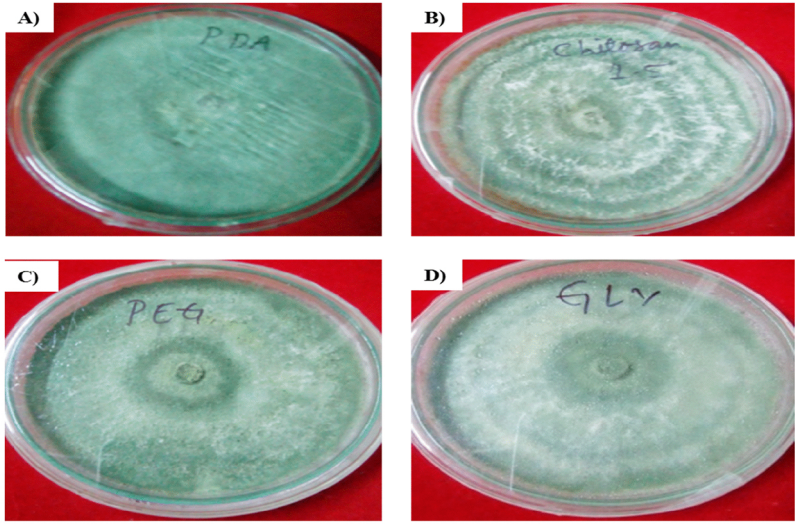
Values in parentheses indicate angular transformed values of germination percentage. When $p < 0.05$ ANOVA Tukey statistical test (95% confidence interval) was performed. Means within a column followed by different letter are significant at 5% level of significance and those following by the same letter do not differ significantly at 5% level of significance.

*T. harzianum* strain no. Th4d; *T. asperellum* strain no. A5; *T. asperellum* starin no. N13

**Highlights:**

- Films were prepared by combining chitosan, PEG and glycerol along with *Trichoderma*
- Seed coating properties and growth paramaters in coated castor seeds was evaluated.
- Tested activity against phytopathogens, compatability with *Trichoderma harzianum*
- Chemical characterization of blend was done by FTIR, TGA, DSC, XRD, SEM etc.
- Chitosan-PEG blend showed advantages in seed coating with antimicrobial activity.

i.




ii.

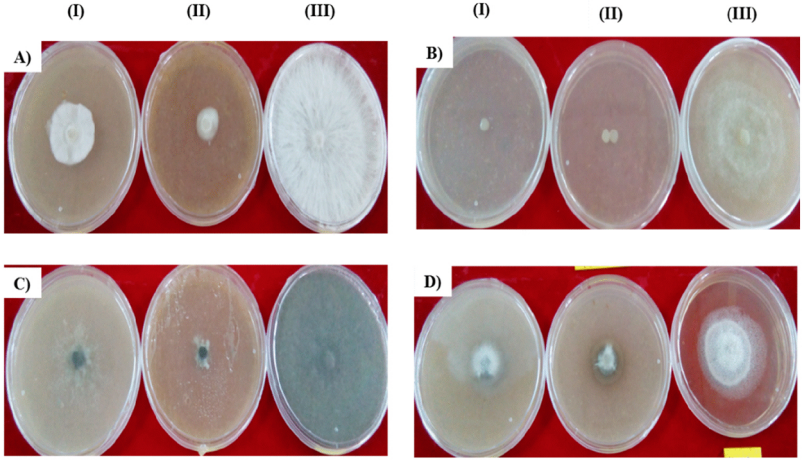



Figure 1

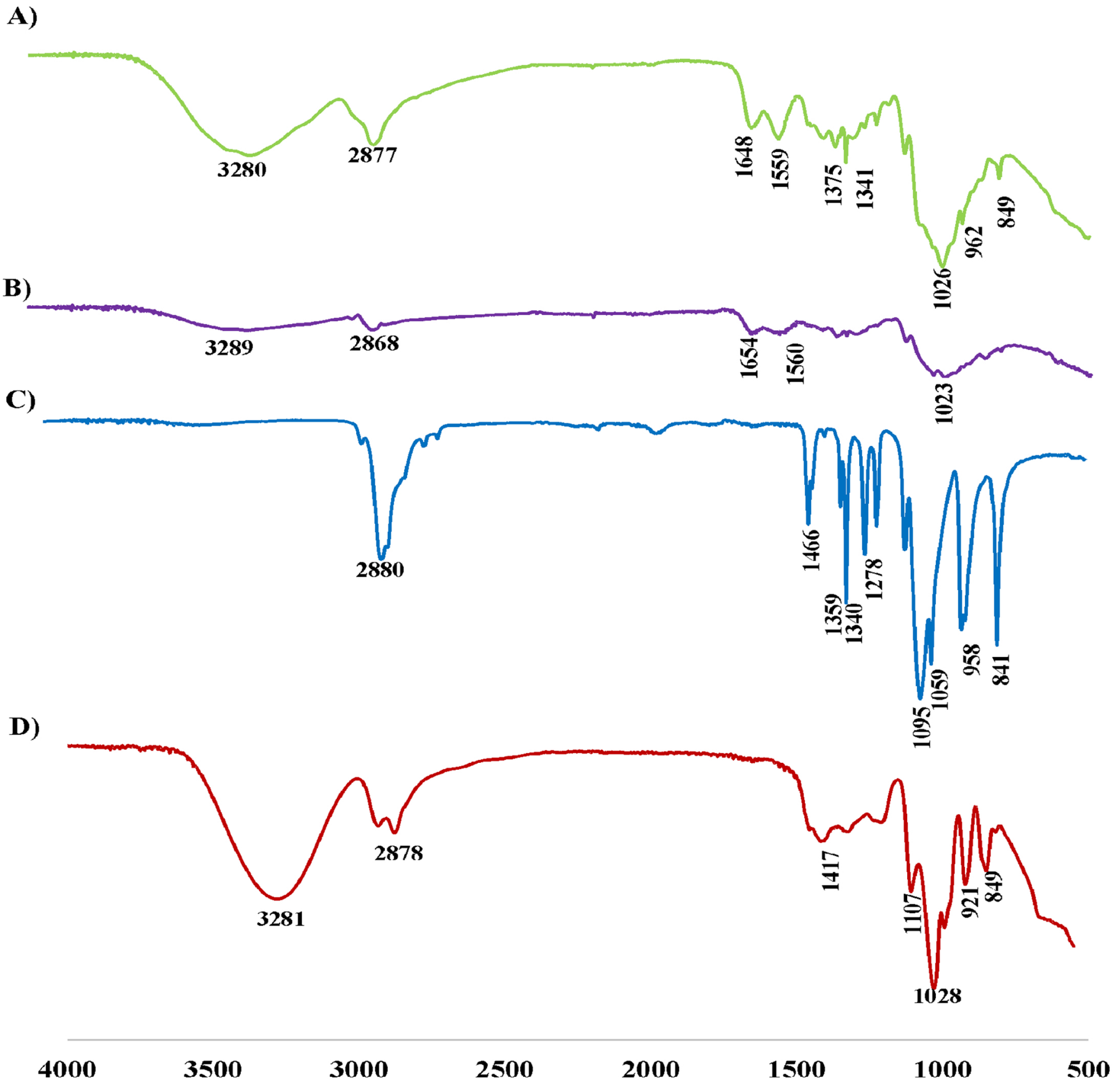
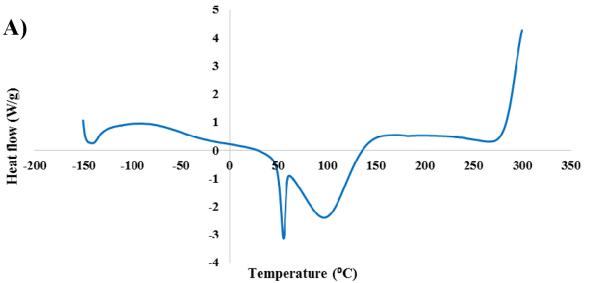
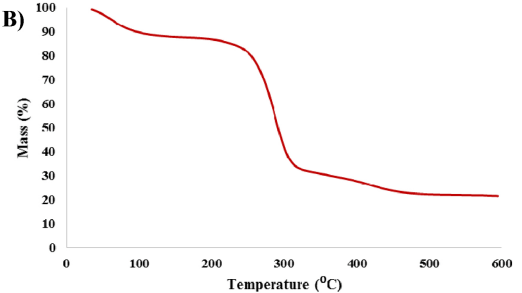

A)
3280
2877
1648
1559
1375
1341
1026
962
849
B)
3289
2868
1654
1560
1023
C)
2880
1466
1359
1340
1278
1095
1059
958
841
D)
3281
2878
1417
1107
1028
921
849
4000
3500
3000
2500
2000
1500
1000
500

Figure 2


A)
Heat flow (W/g)
5
4
3
2
1
0
-1
-2
-3
-4
-200
-150
-100
-50
0
50
100
150
200
250
300
350
Temperature (°C)


B)
Mass (%)
100
90
80
70
60
50
40
30
20
10
0
0
100
200
300
400
500
600
Temperature (°C)

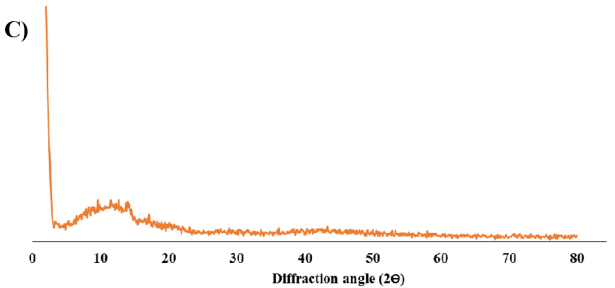

C)
0
10
20
30
40
50
60
70
80
Diffraction angle (2Θ)

Figure 3

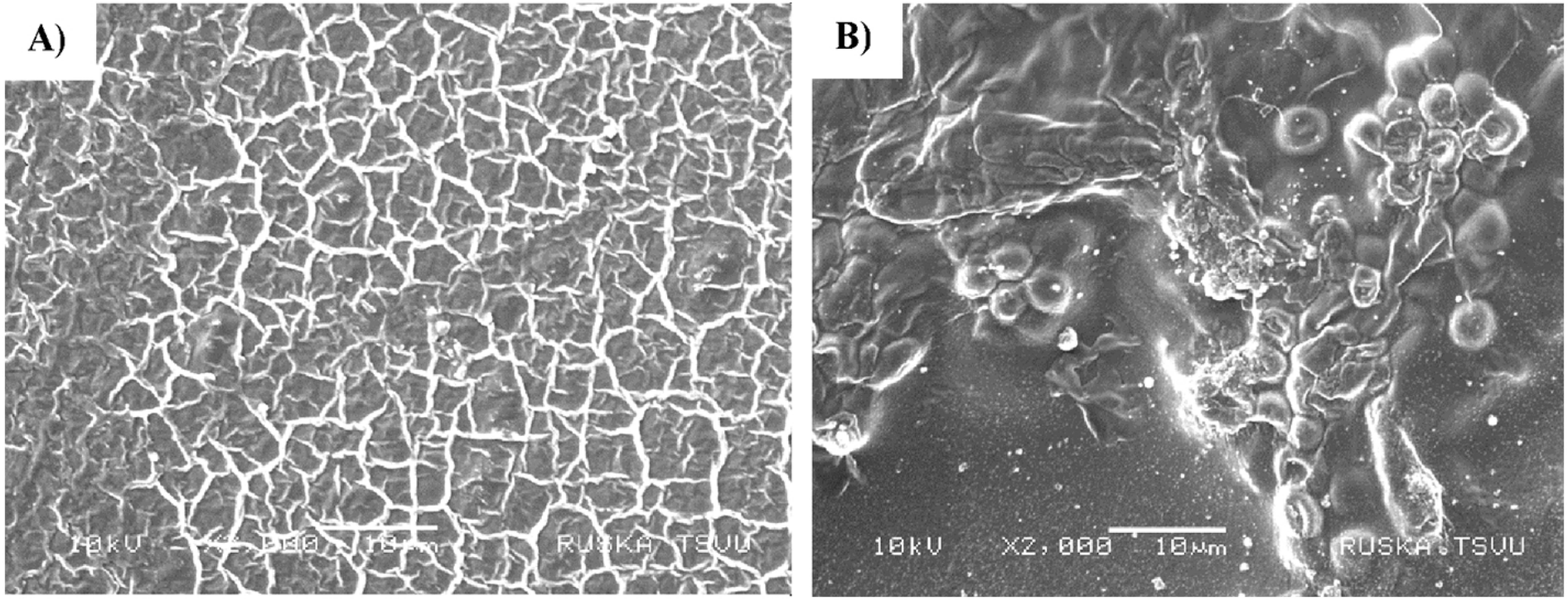



Figure 4

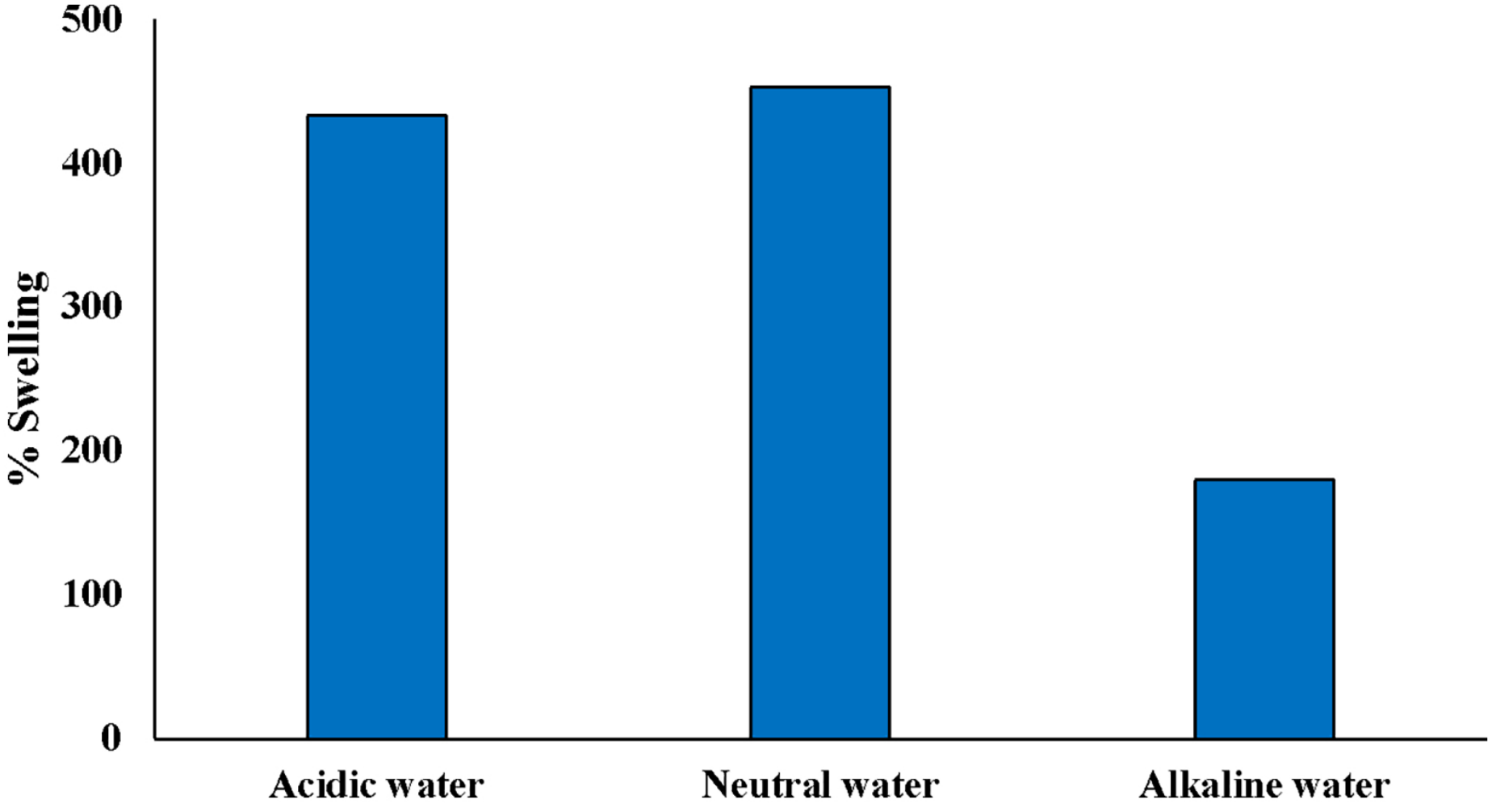

500
400
300
200
100
0
% Swelling
Acidic water
Neutral water
Alkaline water

Figure 5

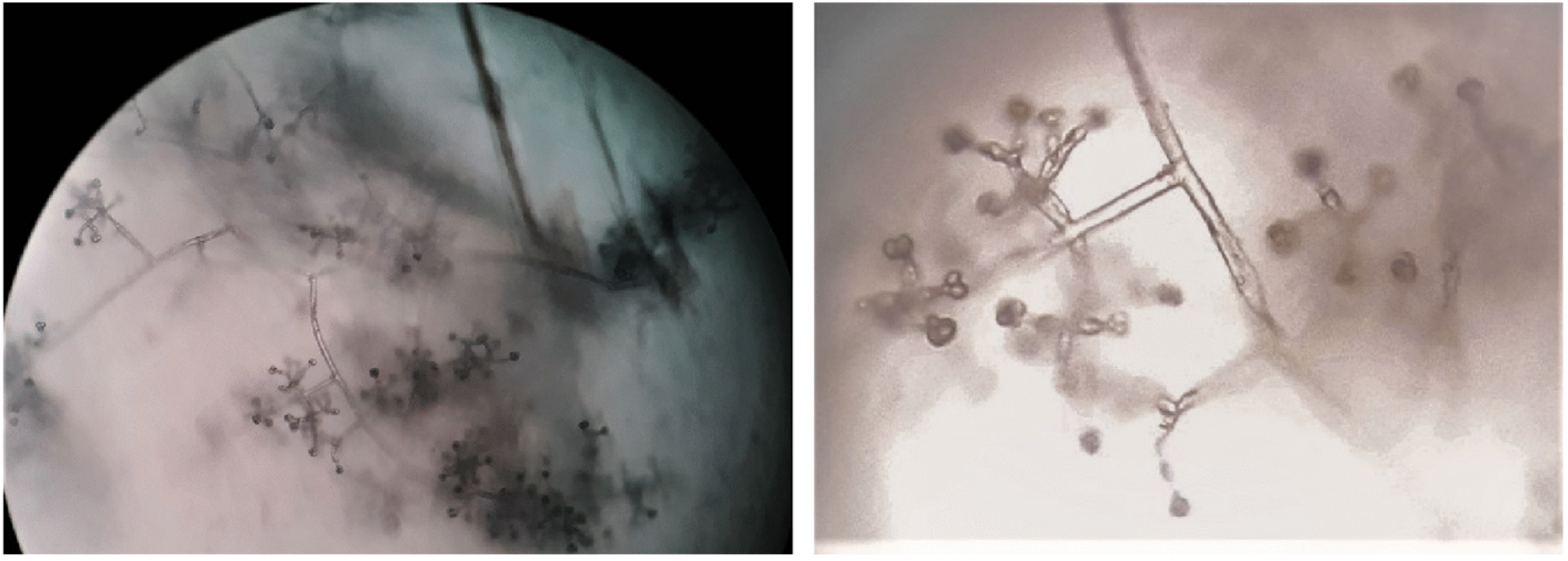


Figure 6