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Methylorubrum aminovorans infused Chitosan/PVA composite nanofibre seed coating to improve germination and seedling vigour in cotton

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Abstract
Cotton is an important fibre crop mostly cultivated under rainfed ecosystem in India. There are many factors limiting the productivity in which poor germination and seedling establishment are the main constraints that reduce the productivity. In order to improve the germination and seedling vigour, a novel seed coating technology was developed using E-spin nanofibre polymer matrix. Herein, a composite nanofibre matrix was developed using Chitosan and Poly Vinyl Alcohol (PVA), in which the plant growth promoting microbial cells (*Methylorubrum aminovorans)* were immobilized. The developed nanofibres were characterized under Scanning Electron Microscope (SEM) and Fourier Transform Infrared Spectroscopy **(**FT-IR) for confirmation of microbial cell loading. The bacterial cells incorporated chitosan composite nanofibres were coated over cotton seeds and tested for seedling quality evaluation. The result of SEM images showed increase in fibre size from 138.2 nm (before loading) to 358.4 nm (after incorporation of microbial cells). FT-IR results confirmed the perfect blending and interaction of Chitosan/PVA molecules with *Methylorubrum aminovorans.* The results of bio-efficacy study revealed that microbial cell loaded nanofibre coated seeds exhibited higher seed germination, seedling vigour and biomass as compared to the control. This study concludes that microbial cells could be immobilized in nanofibre and used as potential seed coating for improving the seed quality.

Keywords: Cotton, *Methylorubrum aminovorans*, Chitosan/PVA, nanofibre, seed quality

1. Introduction
Cotton (*Gossypium* spp.) is an important fibre crop being cultivated in India for the sustainable economy of the country. India is one of the largest producers of cotton with 70% of area under rainfed condition. Low, erratic and uneven rainfall, high temperature, high evaporative demand and a limited soil water holding capacity which affects the rainfed land. All these factors have a negative impact on germination, plant population maintenance, square and boll formation, lint and fibre quality characteristics which ultimately result in a reduction in yield (Karademir *et al.*, 2011) [11]. The possible way of enhancing the yield through seed enhancement techniques include priming, hardening, encrusting and film coating (Bewley *et al.*, 2006) [5]. In particular, microbial seed inoculation acts as an effective and inexpensive seed invigoration technique to increase seed vigour and the beneficial microbes added to the soil enhance effective rhizosphere and crop establishment. However, poor microbial survival and ineffective colonisation of the host plant are two major challenging factors which affect the productivity (Ma *et al*., 2016) [15].
Recently, nanofibre seed invigoration is an innovative technology where nanofibres are developed from various biodegradable polymers (Krishnamoorthy and Rajiv., 2017) [13]. Electrospinning is an advanced smart carrier technology that has been developed to produce nanofibre in a diameter range of less than 100 nm, having high surface area-volume ratio, high porosity and being used to incorporate and deliver various active molecules at the target site (Kayaci., 2014; Bhardwaj., 2010) [4, 12]. Polymers are used as a carrier material for nanofibre development. It is proved that the biopolymer chitosan is difficult to electrospin because of numerous amino groups which making it challenging to develop into a fibrous structure. So chitosan was mixed with other natural or synthetic polymers (Lee *et al*., 2009) [14]. Synthetic biodegradable polymer Poly Vinyl Alcohol (PVA) has great perspectives for development of nanofibres and having excellent physical and chemical properties (Damasceno *et al.*, 2013) [7].
Generally, Plant Growth Promoting Bacteria (PGPB) supports plant establishment, growth and development directly or indirectly (Rocha *et al*., 2019) [22]. Pink Pigmented Facultative Methylotrophs (PPFMs) are known to improve plant growth by various mechanisms *viz*.,

production of phytohormones, N_2 fixation, nodule formation, phosphate solubilisation and synthesis of siderophore production (Anandakumar., 2021) [2]. Additionally, the production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase induces antioxidant enzyme production which helps in mitigating drought stress (Chinnadurai *et al.*, 2009) [6]. The present study was focused on the encapsulation of microbial cells in electrospun nanofibre to protect and improve their shelf life and also the sustained release of the entrapped inoculants to the target site in order to improve the seed germination, seedling growth and vigour.

2. Materials and Methods

2.1 Materials

Cotton seeds were purchased from The SIMA Cotton Development and Research Association, Coimbatore. The polymers PVA with a molecular weight of 1,25,000 g/mol and medium molecular weight chitosan were purchased. The Pink Pigmented Facultative Methylotrophs (PPFMs) *viz*., *Methylorubrum aminovorans* culture was obtained from the Department of Agricultural Microbiology and it was mass cultured in Department of Nano Science & Technology, Tamil Nadu Agricultural University, Coimbatore.

2.2 Methods

2.2.1 Development of Chitosan/PVA composite nanofibre

The biopolymer solution of Chitosan at 2.5% was prepared by dissolving 2.5 g of chitosan in 2% glacial acetic acid (2ml of acid in 98 ml of distilled water). Synthetic biodegradable polymer polyvinyl alcohol (PVA) at the concentration of 10 % was prepared by dissolving 10 g of PVA in 100 ml of distilled water. The solution was prepared individually and the composite polymer was prepared by mixing Chitosan and PVA in the ratio of 2:8 (Helen Rani *et al*., 2020) [21], then constantly stirred in magnetic stirrer at 230 rpm for 6 hours at room temperature to produce a homogeneous clear solution. The solution was loaded into 5ml syringe and electrospun at constant voltage of 25 kV, flow rate of 0.4ml/h, with tip to collector distance of 14.5 cm. The nanofibres were developed and characterized under SEM and FT-IR.

2.2.2 Preparation of E-spin blend for immobilization of microbial cells

The solution of 5% Chitosan was prepared by dissolving 5 g of chitosan in 2% glacial acetic acid (2ml of acid in 98 ml of distilled water). 20 % polyvinyl alcohol (PVA) was prepared by dissolving 20 g of PVA in 100 ml of distilled water. The e-spin blend was prepared by adding 5 ml *Methylobacterium* with 5 ml of composite polymer (Chitosan/PVA) solution in the ratio of 1:1. The blend was prepared and constantly stirred in magnetic stirrer at 230 rpm for 1-2 hours and then subjected to electrospinning and following the optimized parameters and characterized as above for confirmation of microbial cell loading.

2.2.3 Characterization

2.2.3.1 Scanning Electron Microscope (SEM)

The surface morphology and diameter of electrospun nanofibre was characterized in Scanning Electron Microscope (Quanta 250, FEI, and Netherlands) at the Department of Nano Science & Technology, TNAU, Coimbatore. The stub was used to place the nanofibre using double adhesive carbon tape and placed the sample in the specimen chamber for imaging the sample. Then the topography was observed at different magnifications and the images were recorded.

2.2.3.2 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR is a technique for obtaining an IR absorption sample spectrum with the help of the emission spectrum. For the purpose of identifying unknown materials, this sample pattern reveals sample light absorption that creates a distinctive molecular structure at each wavelength. FTIR is used to measure the infrared spectrum absorbance or emission from samples to confirm the presence and interaction of polymers in the developed nanofibre. At a resolution of 4 cm^{-1}, an average of 64 scans was used to gather spectral data between 400 and 4000 cm^{-1}.

2.2.4 Effect of e-spun nanofibre seed coating on cotton seeds

The E-spun solution was applied electrostatically to the seed surface in vertical electrospinning unit at flow rate of 4 ml/h, 18 rpm was maintained at seed coating drum (collector) and at a constant voltage of 23 kV. The cotton seeds were coated with Chitosan/PVA composite polymer (without infusion bacteria) and e-spin solution at different concentration *viz.*, 5,10,15,20 and 25 ml/kg of seeds. The coated seeds were evaluated for speed of germination (Maguire.,1962) [17], germination (%) (ISTA, 2013) [9], seedling length, Vigour Index (Abdul-Baki and Anderson .,1973)[1] and dry matter production.

3. Statistical analysis The experiment was designed in Completely Randomized Block Design (CRD) and the data were analyzed statistically and interpreted at 5% probability level (Panse and Sukhatme., 1967) [19].

4. Results and discussion

4.1 Development of electrospun nanofibre

Biopolymer chitosan and synthetic biodegradable polymer PVA was used as carrier to produce nanofibres for encapsulation of microbial cells (*Methylorubrum aminovorans*). Chitosan/PVA composite polymer was prepared in the ratio of 2:8 which produced smooth and uniform fibres due to perfect functional group interaction of PVA and chitosan which was standardized by Helen Rani (2020) [21]. For optimization of microbial cell encapsulation study, blending microbial broth with PVA in the ratio of 1:1 which was standardized by Chinna Mukiri (2021) [18].

4.2 Characterization of developed nanofibre

The surface morphology of microbial cell loaded Chitosan/PVA composite nanofibre showed that the diameter of the fibre was in the range of 138.2 nm to 293.2 nm and it was increased from 358.4 nm to 396.4 nm due to loading of *Methylobacterium* (Fig.1). This finding is supported by a study of (Chinna mukiri *et al.*, 2021) [18] in which *Methylobacterium* was immobilized in the electrospun PVA nanofibre and the fibre diameter was increased after infusing microbial cells. According to Umran Duru Kamaci (2020) [10], the average elctrospun nanofibre diameter was increased after the addition of enzyme phytase into PVA/Chitosan nanofibre. Further, the scientific report confirmed that the topography of Polyacrylonitile nanofibre was altered due to the increased fibre size is due to eugenol fortification as the average fibre diameter was increased from 127 to 212 nm after loading (Semmani *et al.*, 2018) [23].

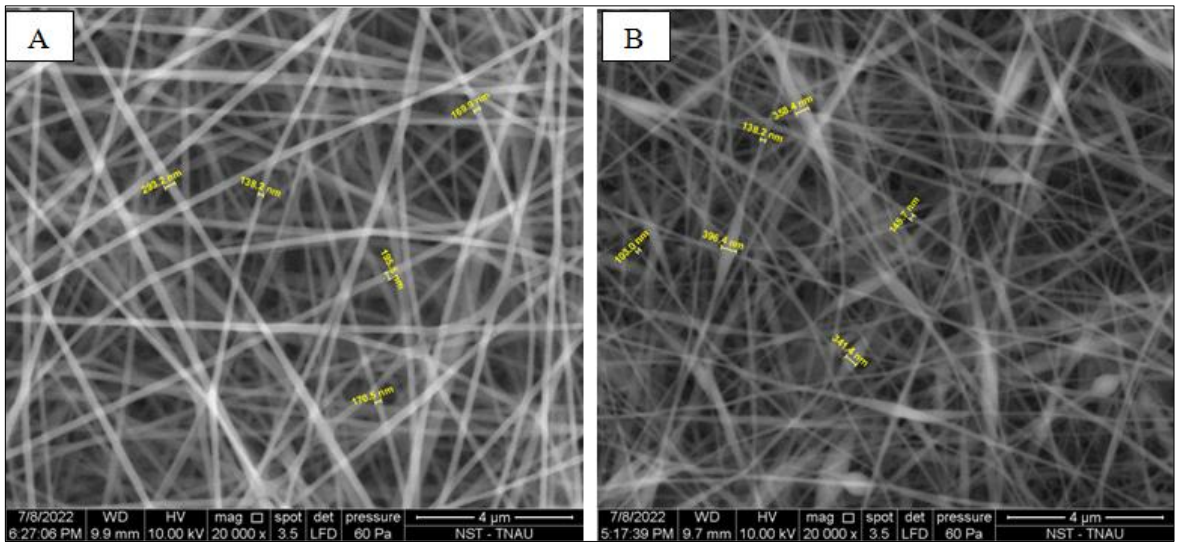


Fig 1: SEM images of electrospun Chitosan/PVA composite nanofibre at 2:8 before (A) and after microbial cells loading (B)

FT-IR results revealed that confirmation and perfect interaction of Chitosan/PVA and microbial infused composite nanofibre. The FT-IR spectra of developed Chitosan/PVA nanofibre was compared with pure chitosan and PVA which confirmed the presence of both chitosan and PVA by exhibiting characteristic peaks at 3338 cm^{-1}, 2903 cm^{-1}, 1636 cm^{-1} and 1377 cm^{-1} due to O-H stretch, C-H stretch, N-H stretch and CH_3 symmetrical stretch. *Methylobacterium* infused Chitosan/PVA composite nanofibre was compared with pure microbial cells and Chitosan/PVA nanofibre by exhibiting characteristic peaks at 3339 cm^{-1}, 2972 cm^{-1}, 1739 cm^{-1}, 1442 cm^{-1} and 1229 cm^{-1} due to O-H stretch, CH_2 stretch, C=O stretch, and C-O stretch respectively (Fig.2). This finding is supported by a study of (Sutka *et al*., 2013) [24] that confirms the interaction of PVA/Cellulose composite nanofibre. Further, the scientific reports confirmed the perfect binding of Ag and ZnO nanoparticles in the Chitosan/PEO nanofibre (Bagheri *et al*., 2021) [3].

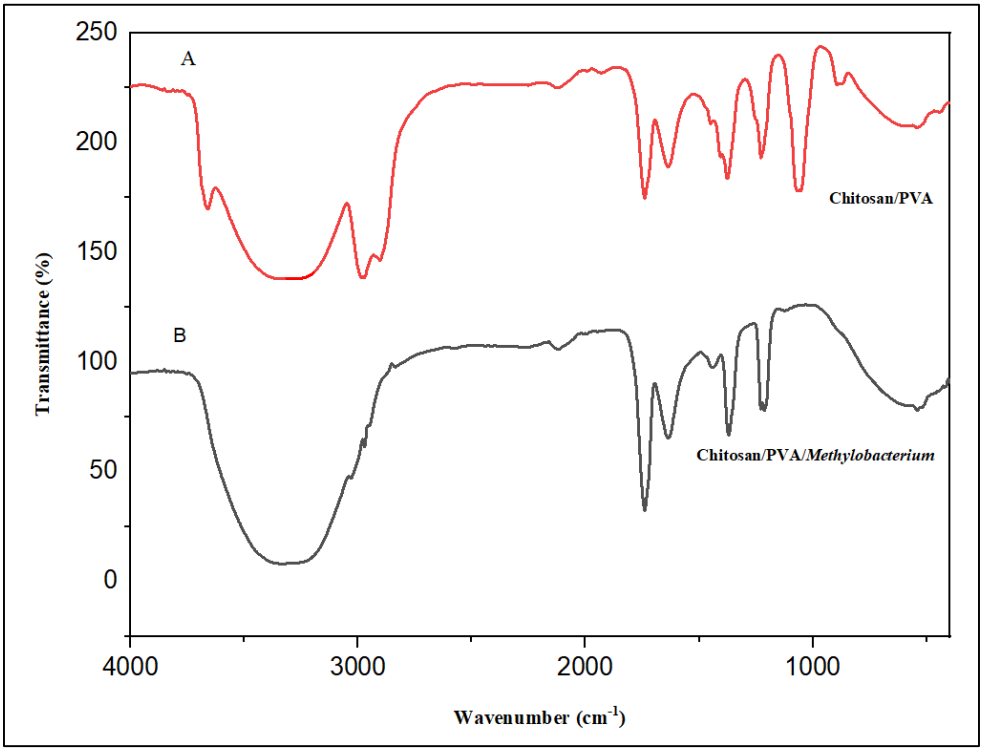


Fig 2: FT-IR spectra of Chitosan/PVA (A) and microbial cell infused Chitosan/PVA composite nanofibre (B)

4.3 Effect of microbial cell immobilized Chitosan/PVA composite nanofibre on seed quality parameters in cotton seeds

The bio efficacy of microbial cell infused chitosan composite nanofibre invigorated seeds performed better than uncoated (control) seeds (Fig.3). Among the nanofibre coated seeds, e-spin blend of 20 ml/kg has significantly recorded the highest germination (78%), speed of emergence (11.33), maximum shoot length (13.6 cm), root length (14.0 cm), dry matter production (0.507g/10seedlings) and seedling vigour (2153) followed by 15ml/kg of seeds as compare to seeds invigorated with Chitosan/PVA composite nanofibre, PPFM inoculated

seeds and untreated seeds (Table 1) & (Fig .4). The improved seed quality is associated to the combined effect of PPFM and the polymer matrix. *Methylorubrum aminovorans* produces phytohormones such as IAA, gibberellins and cytokinins which influence the germination and radical emergence. Further, the high surface area to volume ratio and small particle size improve the bioavailability of the active ingredients that are encapsulated (Madhaiyan *et al.,* 2006) [16]. Invigoration of cotton seeds with hormones and PVP nanofibres led to higher germination and seedling growth under in-vitro condition (Yang *et al.,* 2009) [25]. In addition, there are scientific reports indicated that the beneficial effect of rhizobacteria immobilized nanofibre coating in soybean seeds maintained the bacterial suvival and plant growth parameters (De Gregorio *et al.,* 2017) [8]. The seeds inoculated with *Methylorubrum aminovorans* and PVA electrospun nanofibres recorded the highest germination, seedling length and vigour in groundnut (Chinna mukiri *et al.,* 2021) [18]. Further, the study showed the hormone loaded PVA nanofibre increased germination, and seedling vigour in blackgram and groundnut (Raja *et al.,* 2020) [20].

Table 1: Effect of microbial cells loaded e-spin fibre coating on seed quality of cotton

Treatments	Speed of Germination	Germination (%)	Shoot length (cm)	Root Length (cm)	DMP (g/10 seedlings)	Vigour Index
Control	8.69	62 (51.94)	11.9	11.0	0.419	1438
M. aminovorans inoculated seeds	8.84	67 (54.94)	12.1	11.3	0.430	1555
Chitosan/PVA composite nanofibre coated seeds	8.76	66 (54.33)	12.0	11.2	0.423	1533
5 ml e-spin blend /kg	8.95	68 (55.55)	12.4	12.4	0.445	1608
10 ml e-spin blend /kg	9.25	69 (56.18)	12.9	13.4	0.482	1813
15 ml e-spin blend /kg	10.63	74 (59.34)	13.3	13.8	0.488	2003
20 ml e-spin blend /kg	11.33	78 (62.02)	13.6	14.0	0.507	2153
25 ml e-spin blend /kg	10.24	68 (55.51)	12.4	12.5	0.464	1694
Mean	9.59	69 (56.16)	12.6	12.4	0.457	1725
SEd	0.088	3.028	0.174	0.258	0.007	78.105
CD	0.182	6.249	0.359	0.533	0.013	161.202

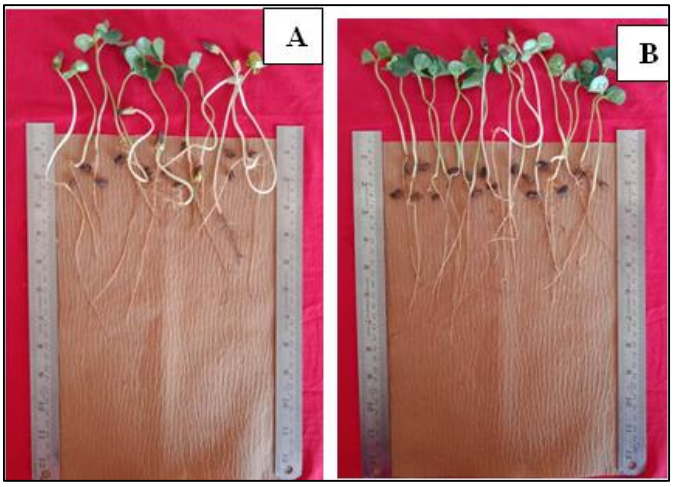
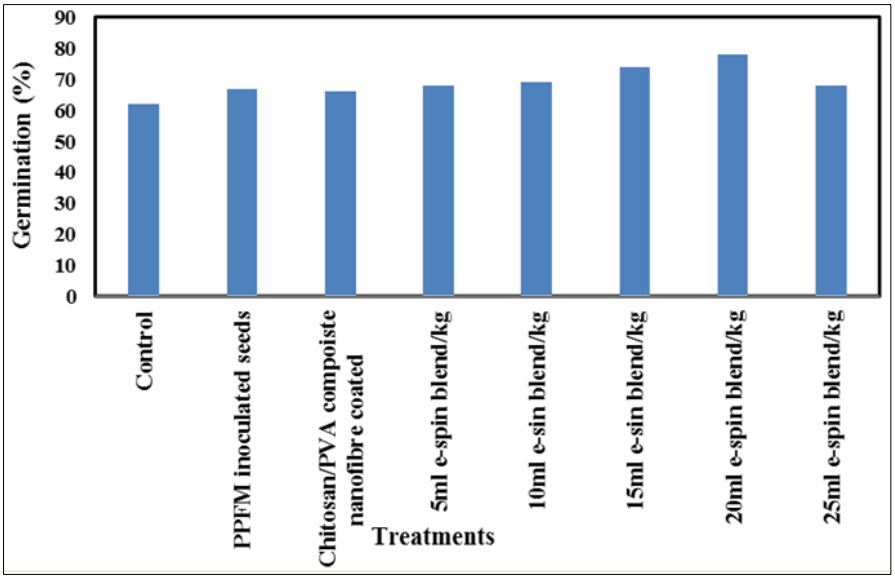


Fig 3: Bio-efficacy test of cotton untreated seeds (A) compared with 20 ml e-spin blend/kg of seeds (B)



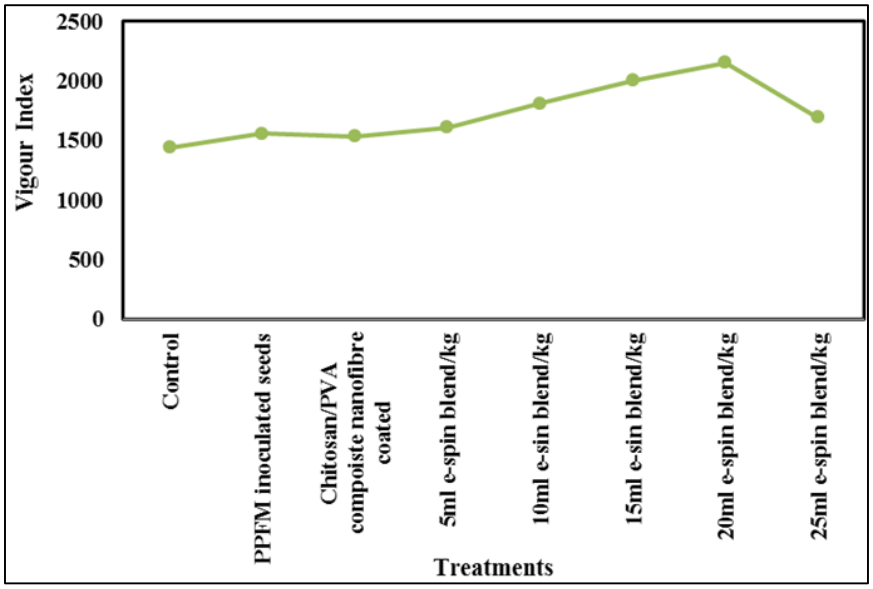


Fig 4: Effect of germination (A) and seedling vigour (B) on e-spun nanofibre coated seeds of cotton

5. Conclusion

The study demonstrated that the composite polymer nanofibre is an effective carrier for successful immobilization of microbial cells (*Methylorubrum aminovorans*) so as to improve the shelf life of microbes and for effective delivery to the targeted site which in turn improves the seed germination, seedling growth and vigour. Since nanofibre seed coating is a novel concept, it requires additional fine tuning, extensive testing, and comparison with currently recommended seed coating treatments in order to gain insight into more advanced techniques.

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