NANOCELLULOSE AS POTENTIAL FILLER MATERIAL, PROTECTIVE SHELL AND ADHESION PROMOTER OF MICROBIAL AGROFORMULATIONS

Claudia PREININGER[1], Ursula SAUER[1], Sara DOPPLER[1], Einav KULBAK[2], Sems YONSEL[3], Carmen Michaela POPESCU[4], Cristina POPESCU[4]

[1]*AIT Austrian Institute of Technology, Tulln, Austria, EU, claudia.preininger@ait.ac.at*
[2]*Melodea ltd, Rehovot, Israel*
[3]*Simbiyotek, Tuzla/Istanbul, Turkey*
[4]*PPIMC Petru Poni Institute of Macromolecular Chemistry, Iasi, Romania, EU*

Abstract

Nanocellulose has emerged as attractive new biobased reinforcement and gas barrier material in for example sustainable food packaging. Herein we report on Cellulose Nano Crystals (CNC) for application in agriculture, as potential filler and adhesion promoter of microbial biofertilizer formulations. Using different types of bacteria we elucidate the physicochemical characteristics of CNC-based microbial seed coatings and spray formulations and show that CNC impacts mechanical strength of seed coatings, but not microbial viability or germination of seeds. CNC is not UV-absorbant except in combination with gelatine, it´s not a good film former, but due to its color it might help to visualize microbial spray pattern.

Keywords: Nanocellulose, microbial formulation, filler, adhesion promoter

1. INTRODUCTION

Most of the biofertilizers and biopesticides are of microbial and biochemical origin (bacteria, fungi, plant extracts) promoting plant growth and resilience or allowing for efficient plant pest and disease management. Compared to conventional pesticides biocontrol agents are usually inherently less toxic and generally affect only the target pest and closely related organisms. Though sustainable and environment-friendly shelf life of microbial inoculants, on-field stability, and active dosage remain critical issues.

Cellulose Nano Crystals (CNC) is a primary building block of the cell wall of plants. CNC has high mechanical strength modulus of 150 GPa and tensile strength of 10 GPA in line with supreme synthetic material and can be used as a nano building block for the enhancement of existing materials and for the production of novel eco-friendly materials [1].

Formulation granting prolonged survival on storage and both survival and sufficient level of bio-activity in field use represents the bottleneck in the broad scale implementation of biological means in plant protection urged by the EU directive on Integrated Pest Management 2009/128/EG. Concerning spray formulations adhesion to the leaves, protection against sunlight (UV), wind and rain and precision of delivery (spraying pattern, spraying volume, time and frequency) are important issues.

In order to tackle these challenges we evaluate nanocellulose as filler in microbial seed coatings for maize and protective shell and adhesion promoter in spray formulations.

2. EXPERIMENTAL

Nanocellulose (Cellulose Nano Crystals - CNC) from pulp (Melodea (**Figure 1**) was added to biopolymer solutions of Gelatine (Sigma Aldrich, Austria), whey protein (Springfield Nutraceuticals BV, Netherlands), milk powder (Carl Roth GmbH and Co. KG, Germany), polyvinylpyrrolidone (PVP), poly vinyl alcohol (PVA) and carboxymethylcellulose (CMC), the latter ones all from Sigma Aldrich, Austria and either used as spray formulations or coated onto maize seeds kindly provided by Saatbau Linz.

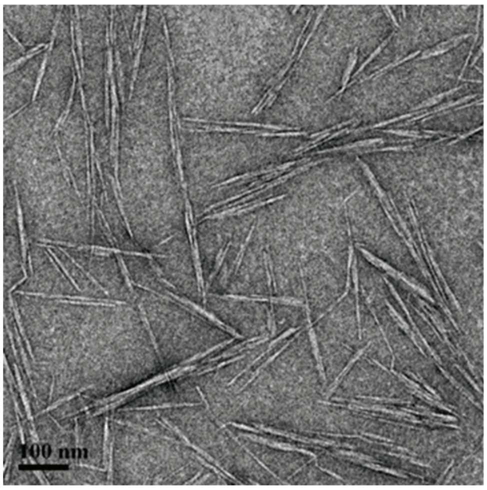

Figure 1 TEM Image of CNC

Bacteria for CNC-based formulations were grown in liquid Luria-Bertani (LB) medium to stationary phase for 72 h as shake culture incubating at 200 rpm at 28 °C. Bacteria were harvested by centrifugation at 4500 rpm and 4 °C during 10 min and resuspended in sterile 0.9 % NaCl before mixed with other formulation components. Number of microbial cells in seed coatings and spray formulations was estimated as colony forming units (CFU) using the serial dilution plate method with nutrient agar medium. Scanning electron microscopy (SEM) scans were imaged with an QUANTA FEG 250 operated at environmental mode (chamber pressure between 30 and 200 Pa), using a GSED Detector and cooling stage.

3. RESULTS AND DISCUSSION

3.1. Nanocellulose as filler in microbial seed coatings

Nanocellulose (CNC) is supposed to increase mechanical strength of biopolymer-based microbial seed coatings and protect the bacteria from adverse environmental conditions during storage. We assume that nanocellulose - similar to nanoclay - attaches to the bacterial cell (1-10 µm) forming a protective shell. To investigate potential benefits of such nanocellulose composite coatings maize seeds were coated with microbial formulations and the different compositions were evaluated with respect to the viability of the bacteria. The bacteria selected include desiccation-sensitive Gram negative *Paraburkholderia phytofirmans*, PsJN (DSM17436) and *Pseudomonas putida* (DSM6125), furthermore robust Gram positive spore-forming strains (*Paenibacillus* sp.). CNC was tested up to 3 % in the coating formulations and at pHs ranging from pH 3 to 5. The coating slurry also contained biobased binder materials like gelatine, whey protein, polyvinylpyrrolidone (PVP), poly vinyl alcohol (PVA), carboxymethylcellulose (CMC) that were tested in both distilled water and 0.9 % NaCl to evaluate the effect of osmolarity on microbial viability. No matter whether spore-forming or non-sporulating bacteria were used microbial viability was significantly (by 2 logs CFU/mL) improved in NaCl-based (about 300 mOsm $\times$ L-1) compared to water-based CNC formulations (between 13 and 22 mOsm $\times$ L-1). Addition of salts also leads to thicker CNC gels and this increased viscosity might also contribute to the enhanced viability. However, film forming capability was improved with aqueous CNC suspensions, the coatings were also more homogeneous, most probably due to better dispersion of CNC.

The seed coating process imposes stress on the bacteria which typically leads to loss of microbial viability during drying. Depending on the type of bacteria - sporulating bacteria are more robust - viability loss might be up to 3 log CFU/mL. To investigate a potentially positive effect of nanocellulose on the desiccation tolerance of bacteria different coating slurries were air-dried in an oven at 25°C under ventilation, rehydrated and the viability determined using agar plate counting. Highest viability was obtained for whey protein-based formulations with and without CNC independently from the type of bacteria used.

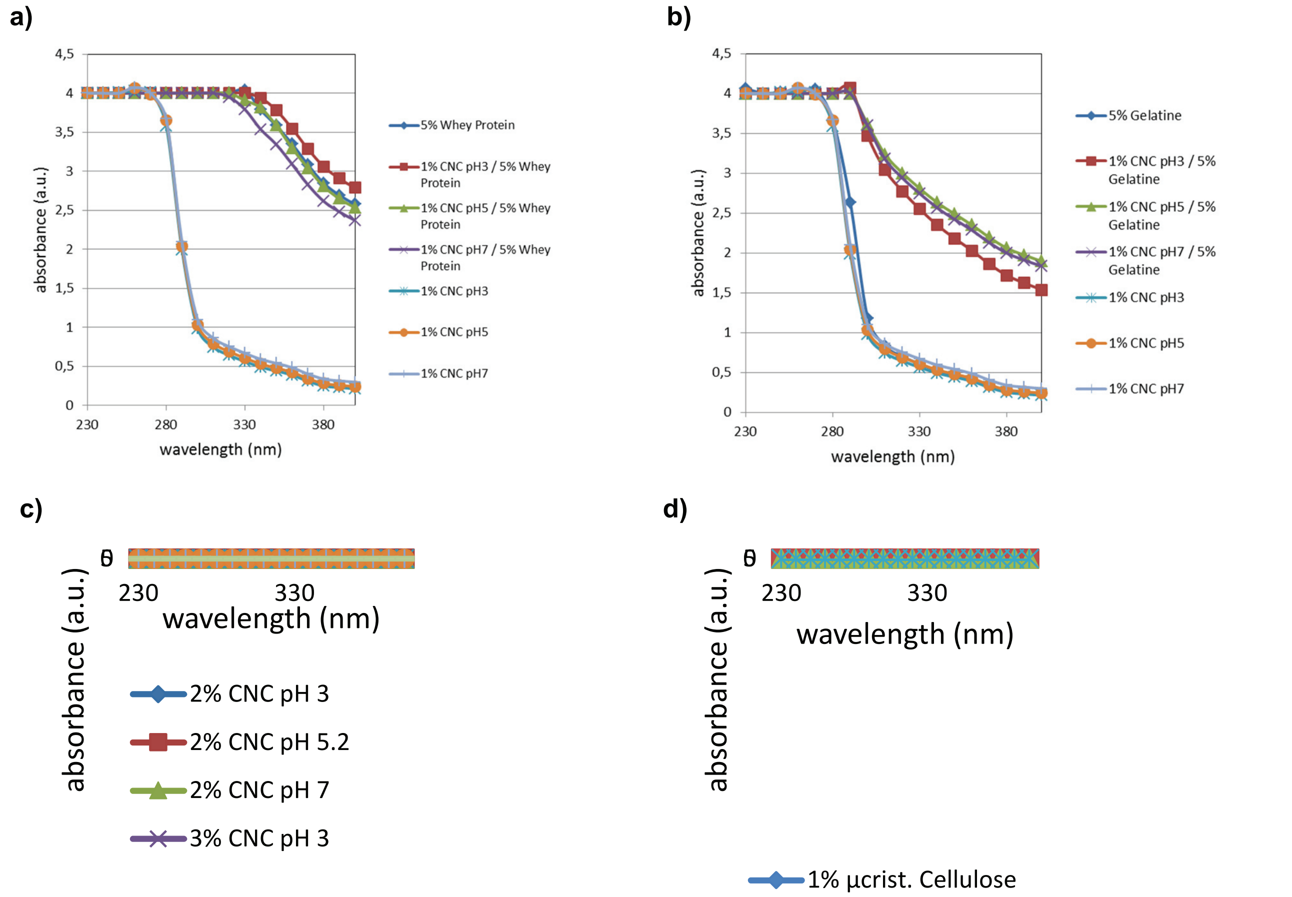

Figure 2 UV spectrum of CNC with a) whey protein and b) gelatine and respective plain solutions, c) the UV spectrum in dependence of pH and CNC concentration and d) different types of celluloses.

Thirdly, the absorbance of seed coating slurries at UV light (280-380 nm) was measured. Protection of bacteria from sunlight to prevent UV damage is generally important, in seed coatings as well as in spray formulations. For this purpose either indicators like congo red, reflectants, such as TiO_2 or lignin-based materials are typically used [2]. Herein we investigate, if nanoparticulate matter like nanocellulose could achieve similar effects. **Figure 2** shows that CNC only weakly absorbs UV-light, independently from the pH (**Figure 2a**), but its absorbance increases with increasing CNC concentration (**Figure 2c**). Compared with other types of celluloses (**Figure 2d**) CNC shows similar UV absorbance than carboxymethylcellulose (CMC), a conventional binder in agroformulations, but less absorbance compared to µcristalline cellulose, often used as absorbent or anti-caking agent.

In combination with stickers a synergistic effect was observed with gelatine: both pure materials do not absorb UV light, while combinations thereof strongly absorb at the relevant wavelengths. This behaviour is in contrast to that of a suspension of CNC in whey protein, where UV absorbance is determined by the properties of the single components only. FTIR studies of nanocellulose, gelatin and their bionanocomposites by Mondragon G. *et al.* [3] reveal that there is no strong intermolecular interaction between gelatin molecules and nanocellulose (no spectral changes in characteristic bands for gelatin and its nanocomposite). Thus it might be more likely that the enhanced UV absorbance of the CNC/gelatin composite is due to the fact that gelatine as the better solubilizer is capable of more uniformly dispersing CNC.

In addition, the seed coating must not prevent the seed from germination. A cold test was performed to evaluate seed vigour under stress conditions and to examine whether the CNC-composite coatings negatively influence germination. The outcome clearly indicated that seed germination was not prevented by any of the CNC-based coatings.

3.2. NANOCELLULOSE AS ADHESION PROMOTER IN MICROBIAL SPRAY FORMULATIONS

In foliar spray development & application not only the microbe-material interaction, but also the interaction of the formulation with the plant leaf surface plays an important role. Adhesion and spreadability of formulations on leaves is of utmost importance, especially at early growth stages where the plant surfaces are very waxy and water-repellent; furthermore for effective spraying good adhesion must be guaranteed to prevent wash-off during rainfall or wind. In order to meet these challenges we aim to use nanocellulose as adhesion promoter, and also for visualization of the spraying pattern on the leaf. **Figure 3** shows the contact angles of 9 formulations including the reference treatments. In **Table 1** the composition of the spraying suspensions and reference treatments (no. 1, 2) are listed. Formulation 3 to 7 were all CNC-based, formulations 6-9 contain (bio)surfactants. A contact angle below 90 °C was defined as a measure for good spreadability of the drops. Results depicted in **Figure 3** show that contact angles of formulation 4-9 were significantly reduced compared to the non sprayed leaves (no. 1). This clearly demonstrates that films of formulation 4-9 are still present on the maize leaves several days after spraying.

Table 1 Composition of selected formulations

No.	Spray formulation
1	no spraying
2	water
3	0.1 % CNC
4	0.1 % CNC, 5 % whey protein
5	0.1 % CNC, 5 % milkpowder
6	0.1 % CNC, 0.1 % biosurfactant
7	0.1 % CNC, 0.1 % commercial surfactant
8	0.1 % biosurfactant
9	0.1 % biosurfactant, 0.1 % talc

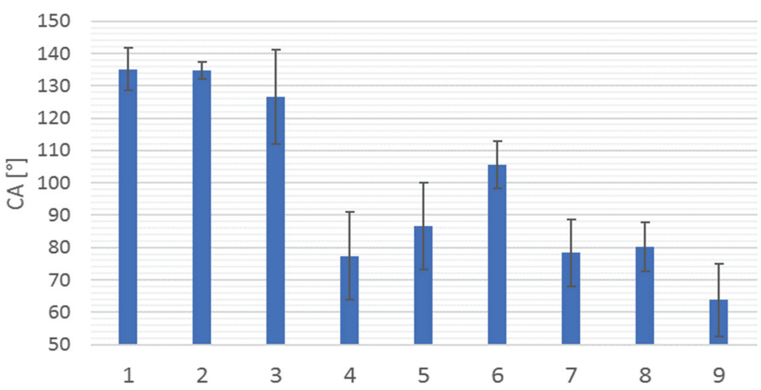

Figure 3 Contact angle of leaves 5 days after spraying with formulations 3-9, and the two reference leaves (no. 1, 2) (mean +/- SD, n=6)

SEM was used to characterize the film sprayed onto the maize leaves (**Figure 4**). High resolution scans of the control leaves show the distinct wax features on young maize leaves causing the high water contact angle of the plant material. With aging the leaves become more hairy and the wax features less prominent.

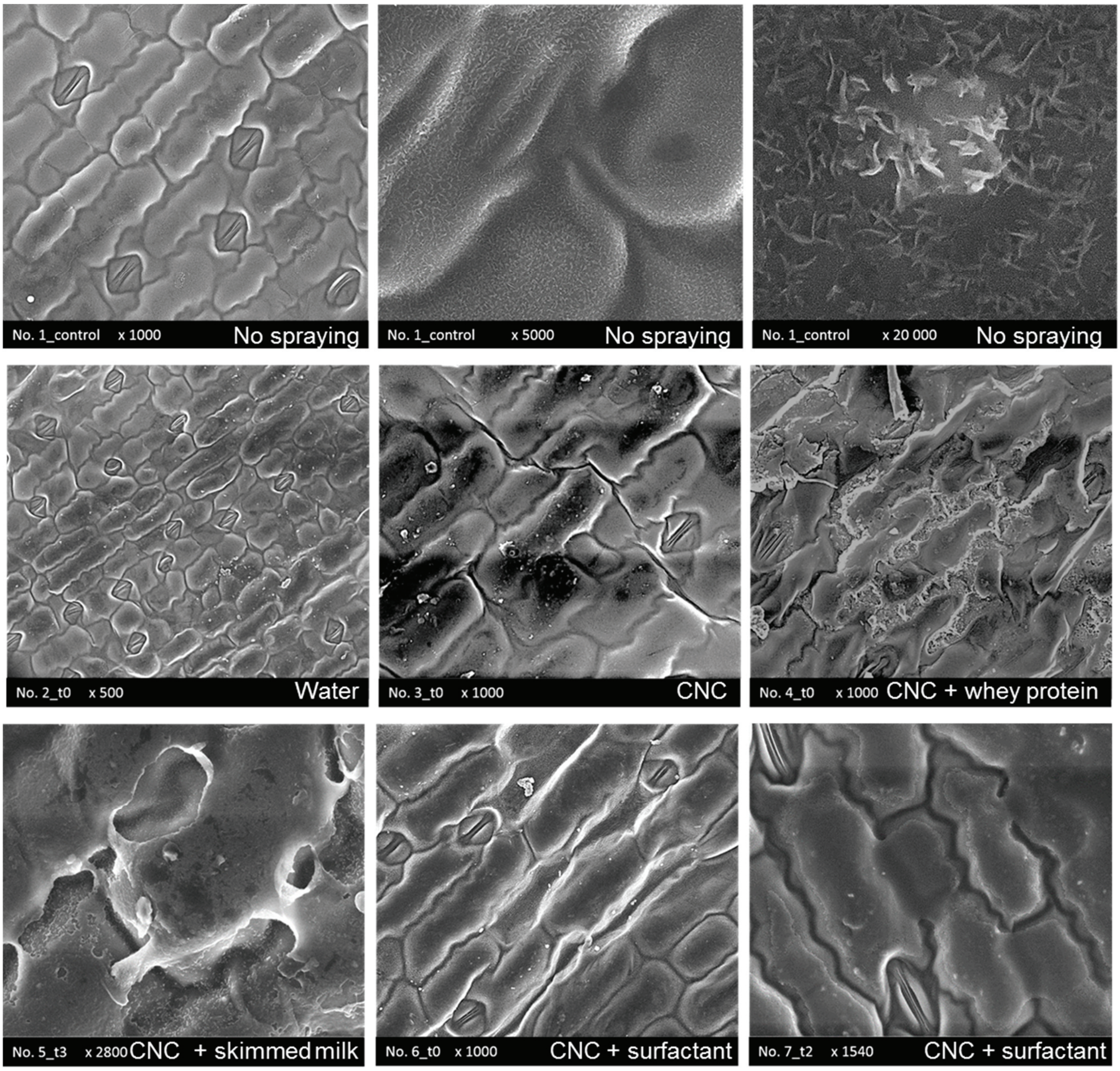

Figure 4 SEM images of plant leaves after spraying, and reference leaves (no. 1, 2)

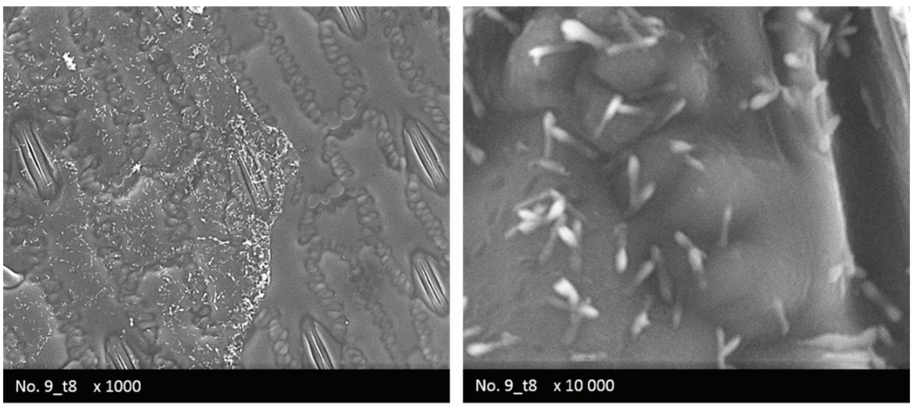

Figure 5 Film of formulation no. 9 (left) with bacterial lawn of rod-shaped bacteria PsJN (right)

Reference leaves in **Figure 4**, show the maize leaf surface compartments including the stomata at 500x to 20000x magnification; as expected there is no film. Leaves sprayed with formulation no. 3 display an incomplete film, only distinct areas of the leaf are covered with spray, but no homogeneous film was obtained. With formulation 4 some crystalline zones are observed. Formulation 5 appears as a thick film on the leaf, while formulation 7 and 8 homogeneously cover the whole leaf surface. Similar to formulations 7 and 8, formulation 6 leads to homogeneous film formation. This clearly indicates that nanocellulose (no. 3) *per se* has no good film forming capability, but with the help of a surfactant fully covers the leaf (no. 6, 7). **Figure 5** shows the film of formulation no. 9 with PsJN after spraying.

4. CONCLUSION

Cellulose Nano Crystals (CNC) used in microbial formulations for agricultural application were shown to improve mechanical strength of seed coatings, but had no protective effect on the selected microbes (microbial viability was not improved). CNC was furthermore tested as additive in spray formulations displaying good spreadability and film forming capability in the presence of a surfactant. UV absorbance was improved in combination with gelatine. Nevertheless lower concentrations of CNC might be more suitable for use with bacteria.

ACKNOWLEDGEMENTS

This work was partly supported by European Commission, Austrian Promotion Agency (FFG), Tubitak, PNCDI III and MATIMOP-ISERD within the m-era.net project COMPIO (www.compio-formulation.eu).

REFERENCES

[1] http://www.melodea.eu/, accessed on 1st October 2018

[2] PREININGER, C., SAUER, U., BEJARANO, A., BERNINGER, T. Concepts and applications of foliar spray for microbial inoculants. Applied Microbiology and Biotechnology. 2018. vol. 102, pp. 7265-7282.

[3] MONDRAGON, G., PENA- RODRIGUEZ, C., GONZALEZ, A., ECEIZA , A., ARBELEIZ, A. Bionanocomposites based on gelatin matrix and nanocellulose. European Polymer Journal. 2015. vol. 62, pp. 1-9.