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SHORT COMMUNICATION

Antifungal Activity of a Fatty Ammonium Chloride Amylose Inclusion Complex against *Fusarium sambucinum*; Control of Dry Rot on Multiple Potato Varieties.

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Abstract

The cationic amylose-hexadecylammonium chloride inclusion complex (Hex-Am) was found to be an effective antifungal treatment for *Fusarium sambucinum* (Fückel), a causal agent of potato dry rot. The Hex-Am treatment was effective against *F. sambucinum* in vitro and in situ, with an effective 50% inhibitory concentration of 400 μg/ml; active component concentration of 20 μg/ml. The amylose complex alone, and blended with polyvinyl alcohol (PVOH), was effective in controlling dry rot in five varieties of potatoes with up to a 99% reduction in damage to the potato tubers. The amylose complex showed no apparent signs of phytotoxicity, with wound periderm reforming within one week of storage at 15 °C and 90% RH. The Hex-Am treatments form an effective antimicrobial film at the wound site, significantly inhibiting fungal damage to the wounded tubers.

Resumen

Se encontró que el complejo de inclusión catiónico amylosa-hexadecilamonio clorado (Hex-Am) era un tratamiento antifúngico efectivo para *Fusarium sambucinum* (Fückel), un agente causal de la pudrición seca de la papa. El tratamiento con Hex-Am fue efectivo contra *F. sambucinum* in vitro e in situ, con una concentración inhibitoria del 50% de 400 μg/ml; con un componente de concentración activa de 20 μg/ml. El complejo de amilosa solo y mezclado con polivinil-alcohol (PVOH) fue efectivo en el control de la pudrición seca en cinco variedades de papa con hasta un 99% de reducción del daño a los tubérculos de papa. El complejo de amilosa no mostró signos de toxicidad aparente, con la reformación de herida del peridermo dentro de una semana de almacenamiento a 15 °C y 90% de HR. Los tratamientos con Hex-Am forman una película antimicrobial efectiva en el sitio de la herida, inhibiendo significativamente el daño por el hongo en los tubérculos heridos.

Keywords Amylose complex · *Fusarium sambucinum* · Antifungal · Potato dry rot

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Introduction

The fungal genus *Fusarium* is comprised of numerous toxigenic species which cause significant yield losses due to plant pathogenic activity and the possibility of contamination of host plants with mycotoxins (Marasas et al. 1984; Munkvold 2017). *Fusarium sambucinum* (Fückel) is a post-harvest pathogen which primarily affects potatoes causing dry rot, a disease responsible for significant yield losses of storage and seed potatoes (Bojanowski et al. 2013). Though *F. sambucinum* is not known to cause toxicoses, it is known to produce numerous mycotoxins: trichothecene, beauvericin, fusarin C, fusaric acid, zearalenones and sambutoxin (Desjardins 2006; El-Hassan et al. 2007; Leslie and Summerell 2008). In particular, it may produce trichothecenes, a group of sesquiterpenes known to cause

toxicoses in plants, farm animals, and humans (Desjardins 2006).

Dry rot is considered both a soil and seed borne disease that requires a wound for entry, since *F. sambucinum* can only infect the potato through a skin rupture (Secor and Gudmestad 1999). *Fusarium* infections normally begin intercellularly, as the hyphae grow between the cells, ultimately becoming an intracellular infection as the fungus penetrates the dead cells (Stevenson et al. 2001). Infected seed potatoes are typically the primary origin of inoculum that contaminates the soil. While *F. sambucinum* can survive in the soil, the infection typically begins after harvest and handling in the presence of contaminated soil (Leach 1985). The disease will then progress during storage, most rapidly at high temperature and humidity but does not spread to non-infected potatoes (Lui and Kushalappa 2002; Secor and Gudmestad 1999). Yield losses can be as high as 25–60%, and are highly dependent on the level of *Fusarium* inoculum in the soil (Chełkowski 1989; Heltoft et al. 2016; Stevenson et al. 2001). Therefore treating the injured potatoes to reduce viable pathogen inoculum and assist in wound healing would allow the tubers to wall off infection sites and prevent the disease from advancing. Treating before storage or up to 10 days prior to planting for seed potatoes can significantly reduce losses from dry rot (Secor and Gudmestad 1999; Wharton et al. 2007).

Potato dry rot is commonly controlled using a number of chemical fungicides, however, increased fungicide resistance has been observed in a number of pathogenic *Fusarium* species (Gachango et al. 2012; Ocamb et al. 2007). Organic certified treatments like copper oxychloride have even been observed to stimulate *F. sambucinum* growth (Baturo-Ciesniewska et al. 2015). Therefore, the utility of a number of disease control agents have been explored, including salts, plant extracts, natural fumigants and biological microbial agents (Baturo-Ciesniewska et al. 2015; Mecteau et al. 2002; Schisler et al. 2000; Vaughn and Spencer 1994). Bio-based polymers can also be utilized, the cationic antimicrobial polymer chitosan has been observed to control potato dry rot from the *Fusarium sulphureum* (=*F. sambucinum*) pathogen, but does display phytoxicity at elevated concentrations (Li et al. 2009).

Amylose is the linear polysaccharide fraction of starch, and is comprised of repeating glucose units with α-(1 → 4)-glucan linkages (Tester et al. 2004). When starch granules are heated in an aqueous solution the amylose will dissolve and can form inclusion complexes with hydrophobic ligands (Godet et al. 1996; Helbert and Chanzy 1994; Obiro et al. 2012). Left-handed helices will form from repeating glucose units, and the helices will have hydrophobic internal cavities, where hydrophobic ligands can reside (Immel and Lichtenthaler 2000; Nimz et al. 2004; Obiro et al. 2012; Saenger 1984).

Amylose-inclusion complexes can be produced by steam jet cooking water dispersions of granular starch, adding the desired ligand to the hot jet cooked solutions, and then cooling the resulting mixture (Fanta et al. 1999). Amylose-fatty ammonium salt inclusion complexes are water soluble film formers which dramatically alter the surface properties of treated cellulosic materials (Fanta et al. 2017; 2013). Blends of amylose-ammonium salt complexes and polyvinyl alcohol produced films with dramatically improved physical properties (Fanta et al. 2016a; 2016b; Hay et al. 2017a). In addition, the amylose-hexadecylammonium chloride inclusion complex (Hex-Am) can be utilized as part of an effective antifungal treatment to protect poplar from wood-decay fungi (Eller et al. 2018).

The objective of this investigation is to determine the efficacy of an amylose-inclusion complex to inhibit the growth of *F. sambucinum* by (1) determining in vitro antifungal characteristics using agar plate studies, (2) determining the in vitro 50% inhibitory concentration of the amylose-complexes, and (3) determining the in situ effect of the amylose-complexes and amylose-complex polymer blends in inhibiting *Fusarium* dry rot damage on five varieties of potato tubers in laboratory assays.

Materials and Methods

Materials

High-amylose corn starch (~68% amylose, AmyloGel 03003) was obtained from Cargill (Minneapolis, MN); hexadecylamine (98%) and hydrochloric acid (HCl, 37%) from Sigma (St. Louis, MO) and polyvinyl alcohol (PVOH, MW 133,000, 99 mol% hydrolyzed) from Polysciences (Warrington, PA). Ultrapure water was used for the preparation of all solutions (Barnstead Nanopure System, ThermoScientific, Asheville, NC).

Preparation of Amylose-Complexes

The procedure for producing the amylose-hexadecylammonium chloride inclusion complexes (Hex-Am) was similar to previous reports (Fanta et al. 2010; Hay et al. 2017b). A solution of hexadecylammonium chloride was prepared by dispersing 5.25 g of hexadecylamine in 217.42 g of 0.1 N HCl, to form the water soluble salt and then heating to 90 °C. High amylose corn starch, 100 g dry weight, was dispersed in 1800 mL of de-ionized water using a 2 L stainless steel Waring blender (Waring Products division, New Hartford, CT). The dispersion was then passed through a Penick & Ford (Penford Corp., Englewood, CO) laboratory model steam jet cooker operating under excess steam conditions (Klem & Brogly, 1981). Temperature in the hydroheater was 140 °C, steam back pressure was 380 kPa, and the steam line pressure was 550 kPa. The starch dispersion was pumped through the jet cooker at a

rate of 1 L/min, and the hot starch solution was collected in a 4 L stainless steel Waring blender container (Waring Products division, New Hartford, CT). The 90 °C solution of hexadecylammonium chloride was immediately added to the starch solution after it was collected from the jet-cooker. The mixture was stirred for 1 min (temperature of 90–95 °C, uncontrolled) and then rapidly cooled in an ice bath to 25 °C. The cooled dispersion was then freeze dried using a Labconco Freezone 6 Liter freeze dryer (Labconco, Kansas City, MO).

Preparation of Sample Solutions

Amylose-hexadecylammonium chloride inclusion complexes (Hex-Am), PVOH and blends of Hex-Am and PVOH were prepared by dispersing the polymers in ultrapure water, heating the dispersions to 80 °C and then immediately cooling the dispersions in an ice bath to 25 °C.

In Vitro Evaluation of Hex-Am on *Fusarium sambucinum*, the Causal Agent of Dry Rot on Stored Potato Tubers

Fungal strain *F. sambucinum* R-6380 was utilized in the bioassay. Conidial inoculum of strain R-6380 was produced in Petri dishes of clarified V-8 juice agar (CV-8) under 12 day^{-1} fluorescent light for seven days at 24 °C, harvested by gently scraping plates after flooding them with weak phosphate buffer (pH 7.2, 0.004% [wt/v] KH_2PO_4 buffer with 0.019% [wt/v] $MgCl_2$), and adjusting the conidial concentration to 5×10^5 conidia/mL using a hemacytometer. Samples of Hex-Am were prepared at a 2X concentration in aqueous solution, i.e. 3% solids Hex-Am. Powders of the dried complexes were dispersed in de-ionized water and heated to 80 °C, and then immediately cooled in an ice bath to 25 °C. All solutions were subsequently filter sterilized using a 0.22 μm MF-Millipore MCE membrane (Millipore Ireland, Tullagreen, IRL).

To determine the influence of these compounds on the viability of conidia of strain R-6380, an aqueous solution of each compound, a weak phosphate buffer control at pH 3.6, or a weak phosphate buffer control at pH 7 was tested individually against a conidial suspension of the pathogen and assayed for effect immediately or after 4 h (Table 1). The pH of one of the control treatment emulates the pH of the Hex-Am (pH 3.6) to ensure that any observed effects were not due to the pH of the solution. To initiate each treatment, 1 mL of a conidial suspension was combined with either 1 mL of a compound or 1 ml of one of the weak phosphate buffer control solutions in 50 mL disposable screw top tubes. The viability of conidia was determined immediately or after tubes were incubated for 4 h (shaker incubator at 25 °C and 125 rpm) by plating treatments on CV-8 and determining colony forming units (CFU/mL) after incubating plates for 3 to 4 days at 25 °C. There were 3 replicates per treatment and the experiment was analyzed as a 2 by 3 factorial with factors of time (0 and 4 h), product (Hex-Am, pH 3.6 water, and pH 7.0 water), and strain R-6380. For each time by product grouping, differences were determined using analysis of variance (ANOVA) and means separated using Fisher's Protected LSD($P < 0.05$).

Table 1 Viable counts of conidia of *Fusarium sambucinum* after minimal and 4 h exposure to Hex-Am amylose complex and pH buffered control treatments in aqueous solutions

Time	Hex-Am	Water pH = 3.6	Water pH = 7.0
0 h	0 [a]	41,000 [b]	43,000 [b]
4 h	0 [a]	53,333 [b]	50,333 [b]

Experiment is a 2 way full 2 by 3 by 2 factorial with factors of time (0 and 4 h), compound (Hex-Am, water pH 3.6, water pH 7), and fungal pathogen (*Fusarium sambucinum* R-6380). Time represents the length of time *Fusarium* conidia were allowed to incubate in each respective product, values for each treatment represent the colony forming units (CFU/mL). The *Fusarium* conidia were exposed to each product and plated after 0 h (immediately) or after 4 h. For each time, values followed by differing letters are significantly different ($P < 0.05$, Tukey HSD; proc. mixed SAS 9.4)

Determination of the IC_{50} concentration of Hex-Am, lowest concentration of Hex-Am that inhibits growth of *F. sambucinum* R-6380 by ≥50%, was performed by testing the optical density of cultures of the strain that were exposed to dilute concentrations of Hex-Am. Strain R-6380 conidia were produced as described earlier and tested against three different concentrations (0.04, 0.008, and 0.0016% (v/v)) of Hex-Am along with a blank RPMI-1640 as a sterility control and a 0% concentration against the R-6380 isolate. The macro-broth dilution was performed following the CLSI standard M27-A3 as the guideline and measuring OD600 on a spectrophotometer.

Isolates

Conidial inoculum of strain R-6380 was grown as described above, harvested from Petri plates using 5 mL of sterile 0.85% saline per plate, vortexed, measured using CrystalSpec Nephalometer, and adjusted as necessary for a McFarland value of 0.5. A working suspension was prepared by diluting the stock solutions for each organism 100-fold into RPMI-1640 (RPMI-1640 medium supplemented with L-glutamine, 2% glucose, and buffered to pH 7.0 with 0.156 M 3-N-morpholinopropane-sulphonic acid (MOPS)).

Preparation of Test Materials Composed of Variants of Amylose Inclusion Complexes

Hex-Am test solutions were prepared as described previously (2.2) and autoclaved as 2% aqueous solutions. A 5-fold dilution of the test material was prepared in RPMI-1640 broth.

Two additional 5-fold dilution series were prepared for the test materials in RPMI-1640. Stock test solutions of 0, 0.4, 0.08, and 0.016% were used for micro-assay to determine growth inhibition.

Determination of Inhibitory Response by Optical Density Measurements

The ability of the test materials to inhibit microbial growth was determined using a broth micro-dilution assay (reference CLSI standard M27-A3). Testing was performed in 96 well flat bottomed plates. Briefly, 20 μL of test solution was pipetted aseptically into designated triplicate wells of 96 well plates. Working suspension (180 μL) of F. *sambucinum* R-6380 was added in triplicate to each concentration of each test material. Optical densities at 600 nm (OD600) were measured on a SpectraMax M2 plate reader (Molecular Devices) at 0 h. The 96 well plates were incubated aerobically at 28 °C for 3 days. After 64 h incubation, OD600 was again measured on each plate to determine if the growth of the microbes was inhibited. Triplicate sterile control wells containing only RPMI-1640 broth for each test material and concentration was tested and remained optically clear.

In Situ *test of Hex-Am on dry rot disease development on 5 varieties of potatoes under storage conditions*

Solutions of 3% solids Hex-Am and 4% solids Hex-Am/PVOH (in a 3:1 ratio) were prepared as described earlier. Conidia of *F. sambucinum* R-6380 were produced as described earlier and set to a concentration of 5×10^5 conidia/mL.

Treatment suspensions consisted of conidia of strain R-6380 mixed 50:50 with Hex-AM or water (as control). Treatment suspensions were used to inoculate 5 different varieties of potatoes (Superior, Russet Norkotah, Russet Burbank, Yukon gold, and Red Norland). Potatoes were wounded with a 2 mm diameter × 2 mm length steel pin. Wounds were then inoculated with 5 μL of the treatment suspension. For each potato variety, each treatment was repeated on twenty four size B, washed seed potatoes (Wisconsin Seed Potato Certification Program, University of Wisconsin Madison, Antigo, WI). Prior to washing, tubers were kept in a cold room at 4 °C and then allowed to acclimate to ~25 °C for 24 h before initiating a bioassay. Each potato received each treatment. Each potato was then placed in a plastic weigh boat containing a dry 2.5 cm square of Wypall paper towel. Boats were moved to trays, the potatoes covered with two dry paper towels, and trays placed in plastic bags. Two additional towels that were moistened with 40 mL of water each then were placed on either side of the tray to promote high relative humidity. The bags were tightly sealed, and then stored for 21 days at 15 °C. Dry rot was then evaluated by slicing lengthwise through the center of each of the wounds. The extent of disease in each wound was rated by adding the greatest depth and width measurements (mm) of discolored necrotic tissue extending below and to the sides of the wound. An initial survey experiment was conducted; preliminary results showed effective antimicrobial activity of the amylose complex polymer blends. A follow up experiment was performed on 5 different varieties of potatoes, 24 potatoes for each variety, and 4 treatments per potato (Fig. 1). For data obtained for each potato variety (24 potatoes per variety), statistical differences were determined using an analysis of variance, and after obtaining a significant F test statistic, the treatment means were separated by the Tukey adjusted least significant difference (Proc mixed SAS 9.4), $\alpha = 0.05$, $n = 24$.

Results

The initial in vitro testing of the antifungal properties of the Hex-Am amylose complexes showed complete inhibition of *F. sambucinum* (Table 1). The pH of the Hex-Am solutions was pH 3.6. Therefore to ensure that the observed antifungal activity was due to the presence of the Hex-Am rather than the acidic pH of the aqueous solution, *F. sambucinum* conidia were also suspended in water without Hex-Am at pH 3.6 and pH 7. There were no statistically significant differences in the *F. sambucinum* CFU/mL recovered after conidial suspension in the two pH treatments. When exposed to Hex-Am, *F. sambucinum* showed a complete lack of growth, with zero observed colony forming units after minimal and 4 h of exposure. To ensure that the effect was fungicidal rather than fungistatic, a portion of the conidia of *F. sambucinum* treated with Hex-Am were twice rinsed with sterile water but removal of Hex-Am from the conidia did not permit conidial germination. To determine the effective antifungal concentration, *F. sambucinum* was exposed to 5-fold dilutions of Hex-Am in a broth micro-dilution assay. The IC_{50}, lowest concentration that Hex-Am inhibits growth of *F. sambucinum* by ≥50%, was determined to be 400 μg/mL. It is important to note that the active component of Hex-Am, the hexadecylammonium chloride ligand, is present at 20 μg/mL or 1/20th the concentration of the starch.

After the in vitro testing, the amylose complex treatments were evaluated on 5 potato varieties to determine their protective properties. The Hex-Am and Hex-Am/PVOH treatments significantly prevented potato dry rot disease damage in all varieties tested (Fig. 1). The Russet Burbank (RB) potato variety displayed the most significant reduction in damage, with up to 99% less damage in the Hex-Am/PVOH treatment as compared with control *F. sambucinum* inoculation. In each potato variety the Hex-Am and Hex-Am/PVOH treatments significantly decreased dry rot damage to the potato tuber.

Each potato variety displayed variable susceptibility to the fungal pathogen, although all varieties showed significant

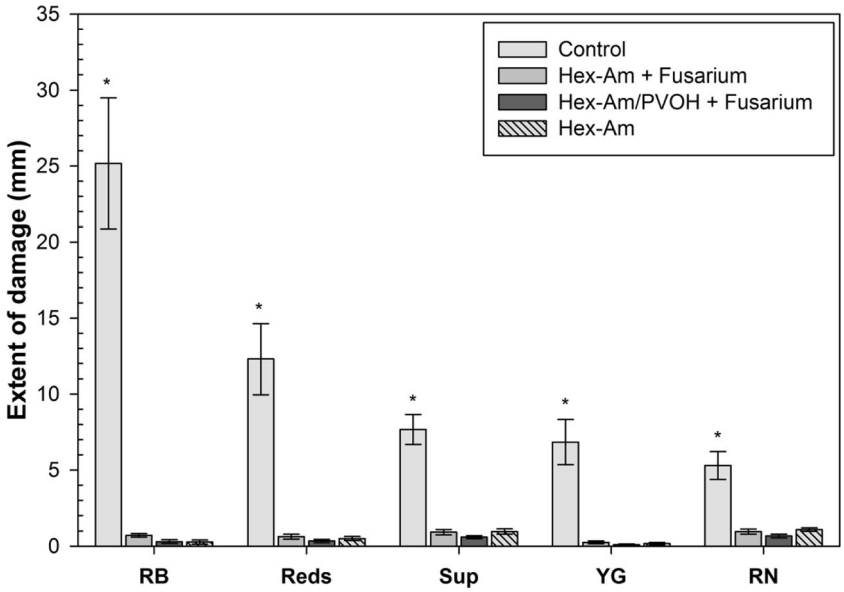

Fig. 1 Extent of Fusarium dry rot disease in inoculated potatoes after 21 days (24 potatoes per variety). Statistical differences were determined using a Tukey adjusted analysis of variance (Proc mixed SAS 9.4), $\alpha = 0.05$, $n = 24$. Error bars represent SE. Note, Hex-Am treatment contained no *Fusarium*; extent of disease is the sum (mm) of the depth and width of dry rot symptomatic tissue around a potato wound. RB = Russet Burbank, Reds = Red Norland, Sup = Superior, YG = Yukon gold, and RN = Russet Norkotah

damage at the control site of *F. sambucinum* inoculation (Fig. 2). Potatoes were assayed approximately 3 months into storage at 4 °C, which may have contributed to a portion of the differential reaction to dry rot seen between the varieties. Russet Norkotah (RN) were observed to be the least susceptible of the tested varieties, generally exhibiting a small shallow infection. The RB variety was the most susceptible, and the fungal infection was able to penetrate deep into the potato tuber (Fig. 2). Even in the RB variety, there was no observed disease in any of the wounds treated with Hex-Am and the pathogen (Fig. 2 B,C). Additionally there was no observed phytotoxicity in the treatment containing Hex-Am alone (Fig. 1, 2d).

Wound sites treated with Hex-Am and Hex-Am/PVOH appear to heal rapidly, as a wound periderm reforms within one week of wounding with no observed phytotoxicity (Fig. 3). The wound periderm can be manually peeled off of the wound site, showing no apparent damage to the tuber tissue underneath from the Hex-Am/PVOH treatment (Fig. 3c).

Discussion

The amylose-hexadecylammonium chloride inclusion complexes were an effective treatment against potato dry rot caused by *F. sambucinum*. Ammonium salts are widely studied for their antimicrobial properties, and primary ammonium salts are utilized in the production of antimicrobial polymers (Kuroda and Caputo 2013; Palermo et al. 2012). Ammonium chloride salt applications on their own do not display any significant inhibitory effects on *F. sambucinum* (Mecteau et al. 2002). Antimicrobial activity typically increases with the addition, and length, of an alkyl group (Palermo and Kuroda 2010). A 16 carbon fatty ammonium salt was selected for this

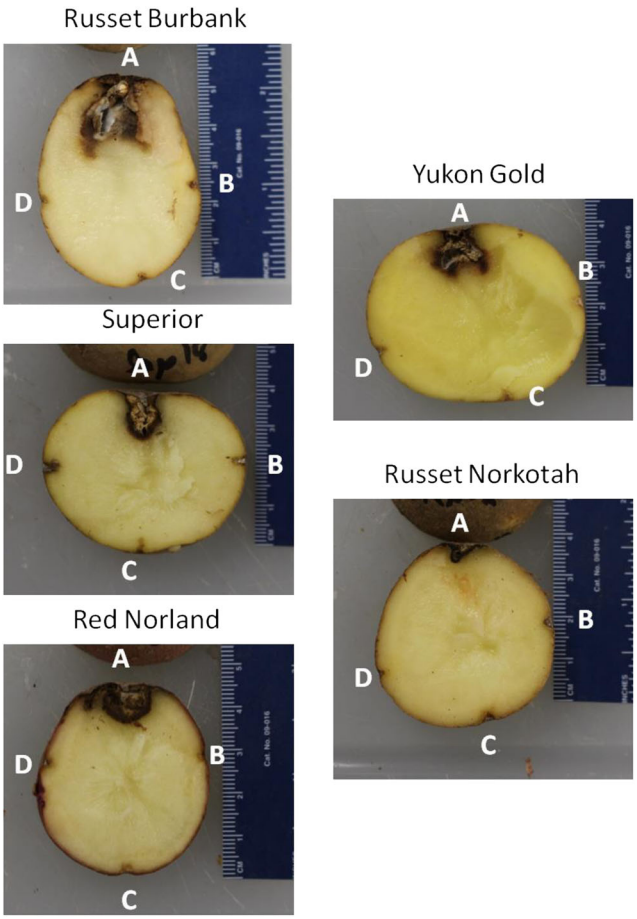

Fig. 2 Representative images of treated potatoes 21 days after treatment. **A**: Fusarium inoculant control and water. **B**: Fusarium inoculate and Hex-Am treatment. **C**: Fusarium inoculant and Hex-Am/PVOH treatment. **D**: Hex-Am treatment alone

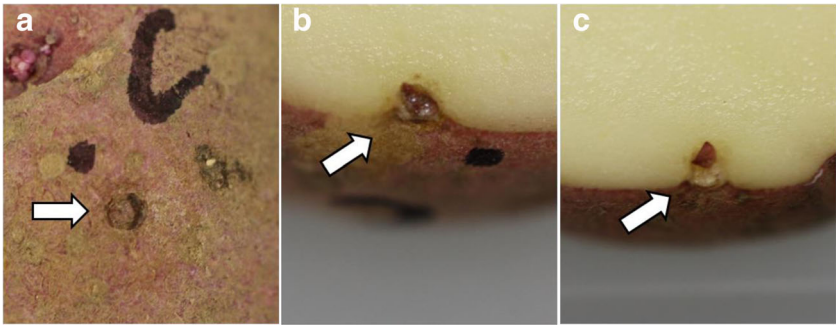

Fig. 3 **a**: Wound site on a Red Norland potato showing wound periderm formation one week after treatment with Hex-Am/PVOH. **b**: Bisection of the wound site shown in image A; showing formation of the wound periderm and no apparent signs of phytotoxicity. **c**: Wound site treated with Hex-Am/PVOH; wound periderm manually peeled off of healing tissue

investigation, but the uncomplexed hexadecylammonium chloride ligand has extremely poor water solubility unless it is incorporated into an amylose complex. Upon complexation, the resulting polymer is water soluble as long as the ligand is charged (Hay et al. 2017a). The novel aspect of this technology is that the incorporation of the hexadecylammonium chloride within the amylose helicies, as verified by XRD analysis (Fanta et al. 2013; Hay et al. 2017b), produces a novel, stable, water soluble antimicrobial polymer with potent antifungal activity. The Hex-Am treatment was effective against *F. sambucinum* with a 50% inhibitory concentration of 20 μg/mL of the active ligand component of the complex. The antifungal properties was observed with the amylose complex alone, and blended with polyvinyl alcohol (PVOH), in five varieties of potatoes with up to a 99% reduction in damage the inoculated potato tuber wound sites.

The Hex-Am amylose complex may be particularly useful because it is a film forming antimicrobial polymer (Fanta et al. 2016a; 2016b; Hay et al. 2017a). Infection occurs at sites of skin rupture. Apart from avoiding tuber wounding all together, wound healing is an essential part of a management strategy for preventing disease and damage to potato tuber (Secor and Gudmestad 1999). Hex-Am has been previously observed to adhere to cellulosic materials, producing significant hydrophobic surface modifications (Fanta et al. 2017; 2013). A film formed at the wound site would assist in protecting the potato from opportunistic infection, while the antifungal properties of the complex inhibits pathogenic fungal diseases. The cationic polymer chitosan can be used to inhibit the growth and development of *Fusarium*, including *F. oxysporum*, but a 50% inhibition of the growth required a chitosan concentration of 1.4 mg/mL, 3.5 fold higher than that of the Hex-Am treatment (0.4 mg/mL) (Al-Hetar et al. 2011). Chitosan has also been utilized to inhibit dry rot disease of potato tubers by *F. sulphureum* at concentration between 0.5–1% (5–10 mg/mL), but concentrations of 1% chitosan were phytotoxic to the potato tuber (Li et al. 2009). Wound sites treated with Hex-Am and Hex-Am/PVOH had no observed phytotoxicity, rather they healed rapidly within a week forming a wound periderm (Fig. 3c). This suggests that the film forming Hex-Am/PVOH treatment may have a temporary bandage like effect of protecting the tuber until the wound can self-heal.

It is expected that this technology could initially be utilized for the treatment of seed tubers prior to planting. The film forming properties of the amylose complex blends would help to protect the susceptible tubers until the wound site is healed. Research is currently underway to characterize the likely antimicrobial mode of action for the Hex-Am treatments, as well as determining suitability for food applications. Alternative bio-based polymers, such as hydroxypropyl methylcellulose, could be blended with the amylose complexes to provide an improved barrier material with variable permeability (Hay et al. 2018). The film forming cationic Hex-Am amylose complex, with and without PVOH, is an effective antifungal treatment to protect potato tubers from dry rot caused by *Fusarium sambucinum*. The Hex-Am treatments effectively inhibit dry rot disease in five varieties of potatoes without apparent phytotoxicity. Additional testing is underway to determine efficacy against a number of potato pathogens, potato tuber wound healing, and suberin deposition in response to a variety of amylose complex formulations.

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