Project Title:

Testing topical application methodologies to induce RNAi for the management of Rhizomania and high-impact soil borne diseases of sugarbeet.

Project Number/Description:

Continuation of a previously funded project.

Project Leader:

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Other Personnel Involved:

Dr. John Weiland, Plant Pathologist

Project Location:

The greenhouse, growth chambers, and laboratory to be used in the proposed project are located at the USDA Northern Crop Science Laboratory, Fargo.

Justification for Research:

Soil-borne diseases account for significant yield and quality losses to sugarbeet growers. Rhizomania disease of sugarbeet, caused by *Beet necrotic yellow vein virus* (BNYVV), is among the most devastating to the crop worldwide (Harveson et al., 2009). Although resistant varieties are currently an important management tool, resistance-breaking strains of BNYVV are present in the growing area and are likely to increase in incidence with continued use of resistant varieties. In Minnesota and North Dakota, which first saw the emergence of Rhizomania in the mid-1990s, Rhizomania has been estimated to reduce harvestable root yields equating to an economic impact of $200 to $300 per acre and cause additional losses through increased transpiration and sugar loss during storage and increased impurities impeding sucrose recovery during processing (Campbell et al, 2008). Additionally, the increase in incidence of Rhizoctonia root and crown rot and the continuing threat of Aphanomyces root rot pose on-going threats to production with minimal available management measures. Losses due to these diseases have been documented in the growing region for decades (Bolton et al., 2010; Harveson et al., 2009). Consideration of these factors in combination with the recent decline in federal funds directed to the investigation of Rhizomania and its management as well as ever-present regulatory pressure on chemical control measures for soil-borne fungal diseases illustrates the need for new approaches, projects, and programs to address this perennial threat to sugarbeet production in Minnesota and North Dakota. Reflecting these concerns, **the current proposal seeks to test novel, yet evidence-based methods for the potential control of Rhizomania and other soil-borne diseases using 'RNA interference' (RNAi).**

Summary of Literature Review:

The induction of double-stranded RNA (dsRNA) that is homologous to all or part of the genome of a pathogen has proven effective in the control of viruses across the plant and animal kingdoms through a process known as RNA interference (RNAi) (Huvenne et al., 2010; Lindbo et al., 2005) This observation has been expanded in recent years to include the control of fungal and bacterial pathogens of plants, and even to insect pests and

nematodes (Duan et al., 2012; Pumplin et al., 2013; Koch et al., 2016). Although RNAi approaches have produced transgenic (genetically-modified; GM) papaya that have, in effect, saved the papaya industry from the ravages of papaya ringspot virus (Gonsalves et al., 2004), GM sugarbeet varieties encoding RNAi-based resistance to Rhizomania (Lennefors et al., 2006; Pavli et al., 2010) currently remain unavailable for sugar production within the US.

Virus induced gene silencing (VIGS) was one of the first exploitations of RNAi in which it was shown that inverted repeat sequences (configured as dsRNA) expressed in a virus and matching that of a cellular gene could suppress the expression of that gene (Lacomme et al., 2003). This observation followed on the heels of experiments indicating that the first GM transgenic plants engineered to resist virus infection were operating through a silencing mechanism (Lindbo et al., 2005). The external application of small stable RNA in the induction of RNA silencing in plants is a relatively recent development (Gan et al., 2010, Robinson et al., 2014) possessing immense potential for the modulation of plant productivity and stress tolerance in the field. Key to the efficacy of the approach is the localized and systemic movement of the silencing signal from the point of application to other areas of the plant (Bennypaul et al., 2012). Although translocation of the RNAi signal is most pronounced using a VIGS approach where the virus carrying the inducing signal is capable of systemic movement within the plant, the spread of small interfering RNAs (siRNAs) produced at a defined location and moving through the plant can occur by other means. For example, Konakalla et al. (2016) recently demonstrated that tobacco mosaic virus (TMV) co-inoculated with dsRNA representing the TMV genome resulted in infection of less than 50% of the plants as compared to inoculations with TMV alone. Additionally, and relevant to the approach proposed here for application of a foliar treatment of dsRNA with the intent of controlling a root disease, it was recently shown that the induction of RNAi in leaves of *Nicotiana benthamiana* (a close relative of tobacco) was followed by silencing of the plant genes in the roots (Bai et al., 2011). Of additional importance to the current proposal is the observation that RNAi, when induced as topically-applied dsRNA, can protect plants from fungal diseases as well. This has given potential to the development

of a management tool called spray-induced gene silencing (SIGS) for crop protection. Notable examples relevant to sugarbeet production is the recent demonstration of the control of Rhizoctonia disease with RNAi (Machado et al., 2015; Tiwari et al, 2017; and Zhou et al., 2016) and the control of the oomycete *Bremia lactucae*, causal agent of lettuce downy mildew (Govindarajulu et al., 2015). *Aphanomyces* and *Pythium* are both oomycete organisms that have infectivity to sugarbeet seedlings and adult roots. The use of SIGS for the management of fungal pathogens and insect pests is a relatively new and exciting area of research in plant protection and an approach that is being pursued aggressively by a number of corporate agri-chemical leaders. Within the scope of the proposed project, therefore, we seek to determine (a) to what extent the use of VIGS or dsRNA applied directly to leaves, beet seed germlings, or during seed priming holds promise for reducing or preventing BNYVV and *Beet soil borne mosaic virus* (BSBMV) accumulation in sugarbeet plants growing in Rhizomania-positive soil and (b) to what degree the approach can be generalized to other soilborne diseases of sugarbeet with high impact to Minnesota and North Dakota production.

Objectives:

New approaches are needed to combat Rhizomania due to a continuing impact of the disease on the crop and the paucity of genes and approved traits for disease management. Expanding on survey work performed by our laboratory regarding the distribution and characterization of BNYVV variants causing Rhizomania, **the proposed project seeks to determine fundamental factors that may show efficacy for controlling Rhizomania, Rhizoctonia root and crown rot, and Aphanomyces root rot in sugarbeet by externally-applied inducers of RNAi.** In light of the need for additional approaches for the management of Rhizomania domestically and the limited federal resources presently directed to research on this important sugarbeet disease, **the objectives for this study within the 2018-2019 funding cycle are**:

1. Test dsRNA in the induction of RNAi directed against BNYVV and BSBMV as a laboratory-generated reagent. If needed, additional tests will be undertaken to deliver

the dsRNA via barley stripe mosaic virus (BSMV) or a disarmed beet black scorch virus (BBSV), both of which produce local infection on inoculated leaves of sugarbeet.

2. Assess dsRNAs targeting the above viruses and as well as key survival genes in *Polymyxa betae* (vector of BNYVV and BSBMV), *Rhizoctonia solani* AG2-2 IIIB and *Aphanomyces cochlioides* for pathogen control through direct application to crowns, roots, and seedlings of sugarbeet.

The assisting scientist, Dr. John Weiland, has several years of experience in working with BNYVV and BBSV of sugarbeet and has spent nearly two decades in in-depth research on the mechanisms involved in plant RNA virus transmission, translocation within the plant, and replication of the viral genome.

Materials and Methods:

Objective 1: Recently it was shown that chemically-synthesized, small hairpin RNA (dsRNA) mimicking the inverted-repeat inducers of RNAi can induce the RNAi state after direct application to plants by spray methods in a technique coined SIGS. Sufficient investigation has occurred in this area to reveal optimized designs for the engineering of synthetic RNA sequence and structure for optimal RNAi induction. In initial tests of the external application of RNAi for the control of Rhizomania via inhibition of both BNYVV and BSBMV, stretches of dsRNA sequence common to both viruses will be applied to leaves and seedlings of sugarbeet test plants (see Fig. 1A). Plants will then be challenge inoculated with BNYVV or BSBMV via mechanical means (Mahmood and Rush, 1999) or transferred to soil containing Rhizomania for BNYVV transmission. Seedlings untreated with dsRNA of BNYVV sequences will serve as experimental controls. Plants will be incubated in Rhizomania-harboring soil for 2-3 weeks after which they will be tested quantitatively for BNYVV accumulation in their root tissue. Quantitative assessment of virus accumulation will be performed using ELISA and quantitative PCR (qPCR) and compared to controls in which no RNAi induction was initiated. As a contingency, if no protection is evident from the RNAi-induced

treatments, then (a) the timing of plant challenge with Rhizomania-positive soil will be adjusted to determine if RNAi induction requires a longer period and (b) the accumulation of 21-25 nucleotide length RNA representing the inverted-repeat sequences, a hallmark of successful RNAi induction, will be assessed in both the leaves and the root tissue of the test plants. Standard methods for the isolation and production of small RNAs have been developed over the past 20 years and are available to the Project Leader and Dr. Weiland.

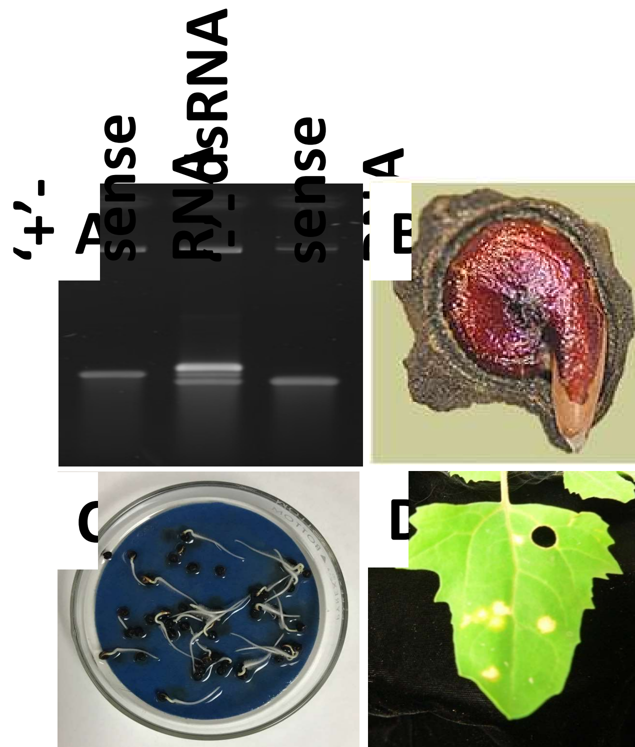

Figure 1. Double stranded RNA (dsRNA, panel A) is synthesized in the laboratory and applied to germinating seeds and seedlings (B,C) as well as to plant leaves (D) in tests of plant protection against viral and fungal pathogens. Photo in B courtesy of Hermann et al. (2007).

Objective 2: Taking advantage of this improvement, modified inverted-repeat structures designed within Objective 1 will be either chemically synthesized by a commercial enterprise or made within our laboratory using standard *in vitro* transcription protocols. The purified synthetic or transcript RNA will be mixed with common adjuvants used in sprays in commercial sugarbeet production and tested as a spray combination or via vacuum infiltration or electroporation of seedlings for the induction of RNAi (Fig 1, B-

D). Seedling root inoculations and treatments will be performed according to Mahmood and Rush (1999) or by planting in Rhizomania infested soil. Challenge of treated plants and testing for inhibition of BNYVV/BSBMV accumulation will be performed as outlined in Objective 1. If necessary, additional adjuvants or RNA-stabilizing supplements added to the commercial adjuvants will be tested for the ability to boost the RNAi effect in the control of virus infection. As in Objective 1, quantitative assessment of BNYVV accumulation will be performed and compared to control treatments in which no RNAi was induced. Additionally, applications will be made to sugarbeet varieties harboring Rhizomania resistance genes Rz1 and Rz2 to assess the added impact of RNAi induction to the reduction of BNYVV in these already-resistant varieties when subjected to heavy Rhizomania pressure in the greenhouse. To expose seedlings to inoculum of *R. solani* AG2-2 IIIB, we will use the procedure described by Bolton et al. (2010). To inoculate plants with *A. cochlioides*, we will inoculate with zoospores as described by Islam et al. (2010). The evaluation of dsRNA for the control of *Polymyxa betae*, the vector or BNYVV, will be incorporated into the results in which plants are protected from disease symptoms and virus accumulation as signatures of Rhizomania disease. **The results of Objectives 1 and 2 lay the groundwork for envisioned potential scale-up of approaches for the control of Rhizomania and possibly other soilborne diseases and pests using RNAi technology.**

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Time Line of Anticipated Accomplishments:

It is anticipated that the objectives outlined above will be met in the 2018-2019 funding cycle.

Project Proposal Budget:

Labor:	$15,000**
Equipment (over $250.00):	----
Supplies:	----
Travel:	----
Leases:	----
Other:	----
Total requested:	**$15,000**

****Note:** The requested labor amount is to support Dr. Weiland, senior research scientist. All additional supplies necessary for the successful execution of this proposal will be provided by the USDA.