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Developing a High-Throughput Method to Screen Soybean Germplasm for Hypoxia Tolerance in Hydroponic Systems

Derrick Harrison
University of Arkansas, Fayetteville

Developing a High-Throughput Method to Screen Soybean Germplasm for Hypoxia Tolerance in Hydroponic Systems

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Derrick Harrison
University of Arkansas
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_____
Leandro Mozzoni, Ph.D.
Thesis Director

_____
Kristofor Brye, Ph.D.
Committee Member

_____
Ryan Dickson, Ph.D.
Committee Member

_____
Larry Purcell, Ph.D.
Committee Member

ABSTRACT

Soybean [Glycine max (Merr.)], the second most planted crop in the United States, is sensitive to field flooding due to depletion of oxygen and accumulation of CO_2 in the rhizosphere. There is a need to breed cultivars that are adapted to areas prone to flooding, but field evaluations in the U.S. are limited because of time (one generation per year) and availability of resources (impermeable soils, irrigation, equipment to build levees). The purpose of this study was to develop and execute a protocol of germplasm screening for hypoxia tolerance using hydroponics in a controlled greenhouse environment. Germination rates and vigor of soybean seeds directly sown onto four substrates were reported using rockwool pellets, perlite, expanded clay pebbles, and a rockwool pellets placed into clay pebbles. Also, a screening protocol was developed consisting of an uninterrupted CO_2 gas treatment at a rate of 200 mL min^{-1} initiated at V2 stage and applied for five consecutive days under hydroponic conditions to produce symptoms akin to those present in flooded soybean fields. Plant responses (normalized difference vegetation index (NDVI), soil-plant analysis development (SPAD), and visual rating) were assessed at termination of treatment and three, six, and nine days thereafter. Such protocol was utilized to screen 34 soybean genotypes of known field reaction, on an experiment that was repeated four times between May and December 2019. Mean NDVI responses differed among genotypes ($p=0.0002$), which were ranked using a Tukey honest significant difference test following application of the predetermined rates and duration of gas treatment. Mean NDVI values ranged from 0.199 to 0.363, with the seven highest ranked genotypes being significantly different than the six lowest ranked genotypes ($p=0.05$). The methodology developed had a high level of repeatability and will help breeding programs screen a larger volume of materials prior to submission for field testing for flood tolerance.

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DEDICATION

To my darling sweetheart, Lena Marie, who has always encouraged and supported me to reach the next level.

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CHAPTER 1: LITERATURE REVIEW

GENERAL

It is estimated that flooding impacts at least 10 %, but upwards of 27%, of all cultivated crop area worldwide with damages exceeding $371 billion annually (Setter & Waters, 2003; Dilley, Chen, Deichmann, Lerner-Lam, & Arnold, 2005; Ward, Pauw, Van Buuren, & Marfai, 2013; Hatfield et al., 2017; Kaur, Priya, Gandhi, & Aggarwal, 2018). To worsen matters, changing climate is altering weather and climate patterns that increase the severity and frequency of severe weather events (Mittler & Blumwald, 2010; Bailey-Serres, Lee, & Brinton, 2012; Voesenek & Bailey-Serres 2015; Alamanos et al., 2019; Aryal,, Shrestha, & Babel, 2019; Aryal, & Zhu, 2019). These flooding events will pose a greater risk to agronomic commodities in the future when there will be a higher demand to accommodate the increasing world population (Normile, 2008; Ringler, Zhu, Cai, Koo, & Wang, 2010). Rhine et al. (2010) reported a 20-39 % yield reduction in soybean due to flooding compared to non-flooded controls. Flood-prone areas, particularly regions that rotate soybean with rice (*Oryza sativa*) may be particularly susceptible (USDA – ARS, 2012). This includes the Mississippi River Delta region (Arkansas, Louisiana, Mississippi, and Tennessee). Given the prevalence of present and potential of future flooding events and the sensitivity of high-value field crops to floods, there is a need to develop flood tolerance via crop improvement (Shabala, 2011; Najeeb, Bange, Tan, & Atwell, 2015; Anderson et al., 2019).

Flooding has long been established as a major yield-limiting factor for commercial soybean production (Stanley, Kaspar, & Taylor, 1980; Oosterhuis, Scott, Hampton, & Wullschleger 1990; Scott, DeAngulo, Wood, & Pitts, 1990; Heatherly & Pringle, 1991; Purcell, Vories, Counce, & King, 1997; Linkemer, Board, & Musgrave, 1998; Sullivan et al., 2001; Mittler, 2006; Ye et al., 2018). Soybean is susceptible to damages resulting from exposure to

waterlogged soils for a duration greater than 48 hours, at which point soils become hypoxic (Griffin & Saxton, 1988; Boru, Vantoai, Alves, Hua, & Knee, 2003). Low-oxygen environments have detrimental effects on plants, including stunted shoot growth and reduction of root growth (Sallam & Scott, 1987; Drew, 1997; Boru et al., 2003); if sustained longer than three days, this may result in plant mortality (Boru et al. 2003; Carlin, 2014). However, tolerance and adaptation to hypoxic environments have been observed in soybean, allowing the plants to overcome and even avoid the stresses of hypoxic environments, thereby limiting impacts on yields.

Soybean is of great economic importance in the United States, as it is the second most planted crop after corn, being grown on more than 30.3 million hectares (75 million acres), with a value of over $31 billion in 2019 (USDA NASS, 2020). The United States was responsible for 123,664,230 of the 348,712,311 tonnes of soybeans produced globally in 2018, ranking first among all countries (FAO, 2020). Soybean is highly valued due to its seed composition, which averages 34.5% protein and 19.6% oil in the United States (USB, 2020). Furthermore, soybean is the primary and preferred global source of plant derived protein for animal feed, with 70% of domestically-grown soybean being used in diets of poultry, swine, dairy and beef cattle, and aquaculture (Erdaw, Bhuiyan, & Iji., 2016; FAO, 2020). The second largest end use of soybean is oil for human consumption, of which soybean is the most important source of edible oils (Wilcox, 2004; Hartman, West, & Herman, 2011). Given the value of the crop, regions that have traditionally grown other row crops have converted to soybean production systems in recent years. This holds true for the Midsouth region of the United States, specifically the fertile Mississippi River Delta region, where soybean cultivation produced yields exceeding the national average in 2019 (NASS, 2020).

Soils and Fertility

Soil properties, specifically texture and structure, are a contributing factor to incidence, severity, and duration of flooding events. Many of the soils in the Mississippi River Delta region are fine-textured, alluvial soils ranging from silty loams to clays, and thus are slowly permeable with poor internal drainage (Scott, DeAngulo, Daniels, & Wood, 1989; Kirkpatrick, Rupe, & Rothrock, 2006). Moreover, soil textures in this region have reduced pore spaces that are readily saturated, thus, rapidly create hypoxic conditions that reduce gas exchange in the rootzone. The reduced aeration leads to anaerobic processes that also affect soil microbiota ratios, soil N phase and soil N content (Van Toai, Beuerlein, Schmitthenner, & St. Martin, 1994; Henshaw, Gilbert, Scholberg, & Sinclair, 2003; Planchet, Lothier, & Limami, 2018). Despite the yield-limiting characteristics of the soil, proper field management and fertility applications may produce exceptional yields.

Soybean requires high levels of N, which has been attributed to seed composition, specifically high protein content (Hurburgh, Brumm, Guinn, & Hartwig, 1990; Roth, Conley, & Gaska., 2014; Gaspar, Laboski, Naeve, & Conley, 2017). Soybean removes large quantities of nitrogen, with nitrogen harvest indices (NHI) that range from 58 to 86% (Pazdernik, Graham, & Orf, 1997; Mastrodomenico & Purcell, 2012; Bender, Haegele, & Below, 2015); therefore, soybean requires fertile soils to achieve the maximum potential yield. A distinctive trait that differentiates soybean from many other row crops is the symbiotic relationship with nodule forming, nitrogen-fixing bacteria in the soil. These bacteria are collectively referred to as rhizobia, the most notable being *Bradyrhizobium japonicum*. There have been several studies conducted that have evaluated the effects that waterlogged and anoxic soils have on root nodulation of rhizobacteria in the rootzone (Bacanamwo & Purcell, 1999a; Thomas, Guerreiro,

& Sodek, 2005; Souza, Mazzafera, & Sodek, 2016). The consensus is that there is a reduction of nodules and therefore nitrogen-fixing activity following a waterlogging event, ergo there is a detrimental effect on yields if N is not managed accordingly.

The presence of N-fixing nodules aids the soybean plant in recovery from flooded conditions (Brandão & Sodek, 2009). It has been observed that soybean plants subjected to hypoxic hydroponic solution will have dramatically and significantly increased uptake of plant-available N in the form of nitrate compared to control soybean plants grown in a normally oxygenated hydroponic solution (Brandão & Sodek, 2009). It is postulated that the reason that plants take up so much N under hypoxic/anoxic conditions is that nitrates can serve as a final electron acceptor in place of, or in concert, with O_2 during respiration (Garcia-Novo & Crawford, 1973). Additionally, nitrate reduction provides reducing power to root cells that could be utilized during glycolysis (Reggiani, Brambilla, & Bertani, 1985; Lambers, Atkin, & Millenaar, 1996).

Flooding and Flood Tolerance Defined

For the purposes of this thesis, the term flood will be used to describe a field in which the water level has exceeded the drained upper limit, also known as field capacity or point of saturation. Water levels exceeding field capacity may be described as waterlogged, whereas water covering any part of a plant above the soil line may be referred to as submergence (Striker, 2012). Van Toai et al. (2001) denoted that there are two classifications of natural flooding: the first of these occurs when a body of water overflows its banks onto a plain that is ordinarily dry, and the second classification of lowland flooding occurs when a plain becomes inundated with water as a result of poor surface or internal drainage. A rhizosphere can undergo a change from aerobic to anaerobic in as little as 24 h when temperatures are

warm and soil microbiota activity is high (Erdmann & Wiedenroth, 1988; Good & Paetkau, 1992). For a flood to become critical and a threat to the health of a crop, duration must last two days or longer (Boru et al., 2003; Sullivan et al., 2001). Hypoxia is a state observed in fields where damages related to water stress occur when O_2 levels in the environment are suboptimal for organisms to carry out normal physiological function (Jitsuyama, 2015). It was recorded that hypoxia occurs when a water-vapor saturated gaseous environment reaches the critical oxygen pressure of ~10%, or ~30% in a circulating solution (Saglio, Rancillac, Bruzan, & Pradet, 1984; Thompson & Greenway, 1991). Hypoxia was measured previously in fields utilizing high-performance liquid chromatography (Araki, 2006; Jitsuyama, 2015), but is measured in greenhouses using a handheld dissolved oxygen meter (Boru, 2003; Araki, 2006). Alternatively, anoxia, a sub-optimal level of oxygen, occurs when ATP produced by oxidative phosphorylation is surpassed by the amount of ATP produced by glycolysis and fermentative pathways (Pradet & Bomsel, 1978).

Plants have adapted morphological and metabolic mechanisms that allow tolerance of hypoxic conditions, specifically aerenchyma and anaerobic respiration (Striker, 2012; Mustroph, 2018). Multiple reports have indicated the presence of genetic control and genetic variation for the traits that allow for a plant to withstand or readily recover from hypoxic conditions (Van Toai et al., 1994; Zhang, Van Toai, Huynh, & Preiszner, 2000; Van Toai et al., 2001; Nguyen et al., 2012; Suematsu, Abiko, Nguyen, & Mochizuki., 2017). There is some inconsistency regarding the description of plants that are adapted to flooded conditions, encompassing a range of durations and responses. Flood tolerance is defined by the USDA-ARS (2012) as the ability of a plant to survive 10 days of steady flooding during the plant's critical flowering stage, whereas Rosielle and Hamblin (1981) defined flood tolerance as minimized or no yield loss when crops

are exposed to flooded conditions, in which the field is waterlogged or, in extreme cases, is submerged. Furthermore, Wetterauer (1996) defined flood tolerance as simply the ability of a variety to yield well relative to a flood-susceptible cultivar. Zhang et al. (2000) defined flooding tolerance as either minimal reduction of growth under flooding or high production of biomass under flooding. Generally, there seems to be an accord that a given cultivar will either have a minimal loss of yield under flood conditions relative to an unflooded control, or, a given cultivar will survive and yield well relative to a flood-sensitive cultivar when both are subjected to flood conditions.

Causes of Flooding

Flooding is often a problem in areas of the southern USA with shallow water tables, high rainfall, and impermeable soils (Scott et al., 1989). There are many factors that may be directly or tangentially associated with geographic, climatic, or physical properties of an area that becomes flooded. These factors may contribute to an existing body of water spilling out of its banks inundating a field, the rising of shallow water tables, or runoff from adjacent slopes, particularly when combined with snowmelt (Lapenta et al., 1995; Boluwade & Rasmussen, 2015). Features such as heavy soils or hardpans can be especially problematic when occurring in flat, level fields found in lowlands such as the Delta. Such landscapes inhibit field runoff, reduce permeability and therefore infiltration of water into the soil, and in turn increase the duration of a flood event (Stuart-Street & Mathwin, 2003). When irrigation is applied prior to a heavy rainfall event, the soil is already close to saturation, which increases the likelihood of waterlogging or even submergence of the plants (Smedema, 1990; Stanley et al., 1980; Sullivan et al., 2001). Furrow irrigation is the most common irrigation method for soybean production in Arkansas (Hill. Popp, & Manning, 2003) and requires a field gradient. As such, there will be one end of

the field that is lower than the other. These lower field ends are more prone to flooding and waterlogging (Heatherly & Spurlock, 2000).

Rainfall is the primary source of water for most flooding events (NOAA, 2019). Heaviest rainfall often coincides with the earlier part of the growing season in the Midsouth (NWS, 2020). Flooding damages during the early vegetative stage is driven by high rainfall that occurs in the area during the early portion of the production season; 35 cm of rain April-June (USClimateData, 2020). Flooding occurs when the rate of rainfall exceeds the rate of infiltration or runoff, usually due to field grade and soil texture. Following a flooding event, available soil oxygen is respired by plants and soil -microbiota to the point of anoxia, when plants become stressed and display symptoms.

Effects of Flooding

In field conditions, soil O_2 and CO_2 concentrations are dependent on the soil texture, soil water content, amount of decomposable substrate, as well as soil microorganism activities (Dueñas, Fernandez, Carretero, Liger, & Perez, 1995; Bouma, Nielsen, Eissenstat, & Lynch, 1997), and thus are highly variable (Rochette, Desjardins, & Pattey, 1991). Soil CO_2 concentrations in a flooded field have been observed as large as 50% v v^{-1} of dissolved gasses (Ponnamperuma, 1972), and Araki (2006) noted the lowest observed CO_2 partial pressure during the growing season was 20 times greater than the atmospheric level during the same period. Also, microbial respiration will increase the rate at which O_2 is depleted and CO_2 is added to the rhizosphere (Buchmann, 2000; Zhai, Zou, He, Ning, & Xiao, 2012). Carbon dioxide becomes toxic to plants as it accumulates to critical concentrations (Liu, Li, Sun, & Chen, 2010).

There are multiple occurrences during water excess that affect soybean unfavorably. A reduction of *B. japonicum* nitrogen-fixing nodules in the rootzone will result in a slowing or

cessation of atmospheric nitrogen fixation because of low O_2 concentrations (Thomas et al., 2005). Available soil nitrogen undergoes denitrification and will volatilize in an anaerobic environment (Vlek, Stumpe, & Byrnes, 1980; Freney, Trevitt, De Datta, Obcemea, & Real, 1990). The compound effects of these stresses will stunt root and shoot growth, cause leaf chlorosis, necrotize leaf and root tissue, limit pods produced per plant and reduce seed mass, thus diminishing overall grain yield (Miao et al., 2012). Negative effects of waterlogging affect soybean as early as planting, resulting in reduced germination and seedling death (Wuebker, Mullen, & Koehler, 2001; Wu, Chen, Hummer, Zeng, & Klepadlo, 2017).

Damages caused by flooding may be evident in as little as two to three days (Griffin & Saxton, 1988; Boru et al., 2001; Sullivan et al., 2001). A reduction of N-uptake and photosynthesis results in the chlorosis of leaves, characteristic of flooding damages, and is partly responsible for the overall stunting of plants (Kozlowski & Pallardy, 1984; Baryla et al., 2001; Yordanova & Papova, 2007). Mutava, Prince, Syed, Song, & Valliyodan (2015) reported that the accumulation of starch granules and a reduction of stomatal conductance in plants was largely responsible for the inhibition of photosynthesis and decrease of chlorophyll. In addition to lack of respiration, root damage occurs due to accumulation of phytotoxins in the soil under anaerobic conditions (Scott, Ferguson, Hanson, Fugitt, & Smith, 1998; Wu et al., 2017b). These toxins include ethylene, ethanol, acetaldehyde, sulfides, soluble iron and manganese, as well as organic acids: formic, acetic, aliphatic, carboxylic, and lactic acid (Fiedler, Vepraskas, & Richardson, 2007; Kozlowski, 1997; McKee & McKevlin, 1993; Pezeshki, 2001; Pezeshki & DeLaune, 1998; Ponnamperuma, 1984). In addition, insufficient O_2 and depletion of energy reserves will result in reduced root uptake of water, gas, and nutrients (Kozlowski & Pollardy, 1984; Scott et al., 1998; Araki, 2006). Following flood-stress, soybean typically experiences yield loss (Scott et

al., 1989; VanToai et al., 1994; Linkemer et al., 1998; Sullivan et al., 2001; Rhine et al., 2010). Furthermore, if flooding conditions last as little as three days, the most extreme effect on susceptible plants is death (Boru, 2003; Carlin, 2014).

It was previously believed that the primary cause of flood symptoms was a lack of oxygen in the root zone (Armstrong, 1980; Jackson, Drew, & Kozlowski, 1984; Kozlowski, 1984). However, it has since been discovered that flood symptoms are caused by suboptimal levels of O_2 coupled with the presence of CO_2 which results in root necrosis and leaf chlorosis, both of which are symptoms associated with flood damage (Araki, 2006; Bacanamwo & Purcell, 1999b; Boru et al., 2003). Soybean plants grown hydroponically were identified to tolerate limited- and no-oxygen conditions in a greenhouse for 14 days with no effect on survival rates or leaf greenness (Boru et al., 2003). However, when CO_2 was elevated to 45% equilibrium in the rhizosphere, 25% of the soybean plants died (Boru et al., 2003). Similar findings were reported when soybean plants were exposed to oxygen-depleted flooding conditions for 21 days followed by root zone CO_2 exposure (Bacanamwo & Purcell, 1999). Soil CO_2 concentrations in flooded fields have been observed as high as 18% v v^{-1} (Ionnou, Schneider, & Grogan, 1977). Concentrations of flooded field soil in a greenhouse were found to be 25 time greater than atmospheric levels in a greenhouse study (Araki, 2006).

It has been observed that soybean plants have adapted mechanisms to overcome flooded field conditions. For instance, specialized adventitious roots can grow above the soil line to reach surface-level atmospheric O_2, and then transport the absorbed O_2 to the submerged root system (Boru, 1997; Bacanamwo & Purcell, 1999b). These specialized adventitious roots are referred to as aerenchyma. The ability of the aerenchyma to provide O_2 to the root zone allows the production of nodules by rhizobia. An adaptation that has been observed in soybean, as well as

other plants, is the timing of response of stomatal closure following saturation of the root zone (Oosterhuis et al., 1990; Else, Davies, Malone, & Jackson 1995). The closing of stomata reduces the rate of photosynthesis and the need for respiration due to water uptake and elemental nutrient assimilation by roots, thereby slowing the rate of O_2 consumption in the rhizosphere, which in turn, delays damage from hypoxia (Sojka, 1992; Sojka, Oosterhuis, & Scott, 2005).

Many legumes, including soybean, are dependent on the symbiotic relationship with nitrogen (N)-fixing rhizobia to supply much of the N required by the plant. Rhizobia bacteria require large amounts of O_2 to form nodules and carry out the processes to fix N_2 from the atmosphere into a plant available form (Sallam & Scott, 1987). Oxygen becomes deficient in flood conditions, thereby restricting rhizobial activity and limiting plant-available N. This sensitivity of the rhizobia to hypoxia and the critical role rhizobia play in providing plant-available N suggest that legumes may be more sensitive to flood than non-legume crops (Bacanamwo & Purcell, 1999a; Boru et al., 2003; Carlin, 2014). Due to the nature of the physiology and morphological characteristics of the soybean plant, more mature plants, such as growth stage V5 and onward, are likely more susceptible to flooded conditions than less mature plants regarding yield loss (Scott et al., 1990; Linkemer et al., 1998; Ara, Mannan, Khaliq, & Miah, 2015).

Management Practices to Mitigate Flooding Effects

There are many cultural practices that have been previously implemented to curb the effects of flooding on field crops, including subsoil tiles, planting in raised beds, and surge irrigation. The use of tile drainage can reduce saturation in soils with poor hydraulic conductivity or an impervious subsoil barrier, especially during periods of snowmelt and heavy rainfall (Wiskow & van der Ploeg, 2003). It is recommended to plant on raised bed to ameliorate the

effects of excessive soil water (Rao & Li, 2003). Current furrow irrigation practices apply water in levels that exceed crop requirements, resulting in partial field saturation and the accumulation of tail water (Kandpal, 2018); alternative irrigation methods such as surge irrigation can reduce runoff water losses and may help reduce flooding (Nishihara & Shock, 2001). However, the above practices may require specialized equipment or other significant investments on behalf of the farmer. Furthermore, depending on soil texture or severity of a rainfall event, these practices may not effectively prevent yield losses resulting from a flood.

Selection of flood-tolerant cultivars is the best cultural practice to mitigate losses associated with flooding events. Multiple quantitative trait loci (QTL) that confer tolerance to flooding conditions have been identified for various growth stages in soybean (Van Toi et al., 2001; Cornelious et al., 2005; Githiri, Watanabe, Harada, & Takahashi, 2006; Van Nguyen et al., 2018; Hummer, 2018). It has been postulated that yield reductions following flooding could be cut in half by planting flood-tolerant cultivars (Shannon, Stevens, Wiebold, McGraw, & Sleper, 2005). There is a need to develop and increase the number of stable, flood-tolerant lines that are available to the market (Rizal & Karki, 2011; Wu et al., 2017c). When coupled with other cultural practices, an adapted cultivar may reduce yield losses from field flooding.

Phenotyping Tolerance/Susceptibility

Prior experiments have been conducted with the goal of evaluating or screening soybean germplasm for tolerance to hypoxic conditions. Several methods have been employed to differentiate tolerant and sensitive lines. A common approach exploits the change in greenness resulting from decreased chlorophyll content as a response to low-oxygen environments. Bacanamwo & Purcell (1999a), Boru et al. (2003), and Araki (2006) all used a portable chlorophyll meter (Minolta SPAD-502) to measure leaf greenness at intervals following different

durations of low-oxygen treatments. All studies determined that there is a quantifiable decrease in chlorophyll content as a consequence to prolonged hypoxic conditions. Normalized difference vegetation index (NDVI) is a second common measure of plant chlorophyll that utilizes near-infrared light to detect greenness (Plant et al., 2000; Lizaso, Batchelor, & Westgate, 2002). As chlorophyll content decreases there is a relative change in spectral reflectance that can be detected with NDVI but is not perceived by the human eye (Adams et al., 1999). The ability to easily interpret minute changes in canopy spectral reflectance quickly and at low cost makes NDVI suitable for tracking phenological and physiological changes across space and time (Magney et al., 2016; Gamon et al., 2019).

Another approach to differentiate plant response to flooding is use of a visual scale of chlorosis and necrosis damage. Previously-used scales range from five-point to ten-point scales (Cornelious, 2005; Wu et al., 2017b; Wu et al., 2017c). The soybean breeding program at the University of Arkansas was able to categorize 2000 entries over seven years using a visual scale (Wu et al., 2017c). Other traits used to characterize flood response of soybean include plant survival rate (Boru et al., 2003; Araki, 2006; Wu et al., 2017b; Wu et al., 2017c), biomass accumulation (Bacanamwo & Purcell, 1999; Boru et al., 2003; Araki, 2006), number of branches (Boru et al., 2003; Araki, 2006), height, leaf area, root length, root and stem base porosity, and yield (Bacanamwo & Purcell, 1999; Boru et al., 2003; Araki, 2006).

Overcoming Environmental Effects Using Hydroponics and Greenhouses

Hydroponics permit fast growth due to decreased energy inputs from the plant to develop a large root system to search for water and nutrients (Tocquin et al., 2003; Ali et al., 2019), which are amply supplied in a hydroponic system. The hydroponic solution is also capable of providing specific levels of necessary macronutrients and micronutrients that can be tailored to

the crop that it nourishes (Savvas & Adamidis, 1999). Conversely, there are universal nutrient solutions that are not targeted to specific growth stages or crops, one such example is the Hoagland solution (Savvas, 2002). A hydroponic system may be outfitted to infuse atmospheric air into the solution to provide the rootzone oxygen necessary for respiration and gas exchange. The same materials used for infusion of atmospheric air may also be adapted to incorporate other gases into the solution.

An advantage of simulating flooding in a hydroponic system instead of screening in a field is the level of control and repeatability achieved in the greenhouse (Kozai, Kubota, & Jeong, 1997). Another component of field experiments that will be excluded in the hydroponics setup is the element of soil microbiota (Woitke & Schitzler, 2004; Chave, Dabert, Brun, Godon, & Poncet, 2008) Furthermore, O_2 diffuses 10,000 times slower in water than the atmosphere (Colmer, 2003; Wegner, 2010). Also, because of the constant addition of pumped air, a recirculating hydroponic system will be able to provide optimum O_2 to the roots of the soybean, allowing for maximum efficiency of physiological processes where O_2 is the limiting factor. Oxygen from pores in the substrate may be taken up passively by the root tip (Lemon, 1962; Luxmoore et al., 1970; Yafuso & Fisher, 2017). Being able to meter out a predetermined volume CO_2 using a pressure regulator, and to assess O_2 displacement will allow for the optimization of hypoxic conditions. It has been shown in experiments with wheat (*Triticum aestivum*) plants that nutrient solutions depleted of O_2 could reproduce the same effects as soil waterlogging (Guyot & Prioul, 1985).

Studies Evaluating Soybean in Greenhouses and Hydroponics

The previous studies implementing the use of greenhouse or hydroponics have mostly studied how soybean plants respond to hypoxic conditions with little focus on their use for

screening purposes. Boru et al. (2003) evaluated the responses of soybean plants to hypoxic conditions, displacing oxygen with N_2 gas, as well as N_2 gas with increasing proportions of CO_2 gas. Findings indicated that as the proportion of CO_2 gas increases to 50% v v^{-1}, up to 25% of plants died, while surviving plants exhibited chlorosis as well as necrosis of roots and leaves. In addition, total biomass and N accumulation decreased with increasing CO_2 proportions, confirmed by weighing roots and shoots and measuring leaf greenness following treatment (Boru et al., 2003). The work established that soybean damages incurred under hypoxic conditions are more severe in the presence of CO_2. Boru et al. (2003) examined responses of a single cultivar 'Williams', which limited the understanding of how the methodology would effect a range of tolerant and sensitive cultivars.

Araki (2006) further examined CO_2 effects on water uptake as a function of leaf transpiration. Using pregerminated plants of soybean cultivar 'Enrei' grown in field soil media, Arakai (2006) performed gas additions to the rhizosphere and assessed leaf water potential and leaf greenness. Results indicated that CO_2 presence in waterlogged environments reduced stomatal conductance and total N uptake (reducing leaf greenness), thereby reducing total biomass accumulation (Araki, 2006). While these findings are useful for confirming that characteristic responses under waterlogging conditions can be simulated in controlled settings, it does little to test the application of these techniques to identify tolerance across any substantial number of genotypes.

Carlin (2014) developed a method to screen germplasm in a greenhouse setting that relied on the use of potting soil and field soil media submerged in flood water that had an unspecified volume of CO_2 added. The treatment required 14 days from the beginning of treatment at the V3 growth stage to produce symptoms consistent with waterlogging stress. While the method could

be useful to identify tolerant/sensitive germplasm, there is an opportunity to increase efficiency by utilizing hydroponics.

Finally, Jitsuyama (2015) determined the different responses of 12 cultivars grown in hypoxic and aerated solutions; however, the methods did not include the additions of CO_2, instead using only an oxygen absorber. Jitsuyama (2015) concluded that there were no significant differences in plant response between the two treatments for all cultivars, further confirming findings from the Boru et al. (2003) study that CO_2 presence greatly exacerbates damages in low-oxygen environments.

All these greenhouse experiments collectively elucidate the mechanisms by which low-oxygen environments, such as those found in a flooded field, negatively impact soybean. However, there is still the need for a methodology that is of higher throughput for breeding applications. To accelerate screening germplasm for tolerance, we propose that soybean breeding lines or accessions are grown in a deep-water hydroponic system in a greenhouse and their response assessed using NDVI. The proposed system could decrease screening time and resources, and may increase the repeatability of results, all of which could positively affect the heritability and genetic gain for the flood-tolerance trait while reducing the cycle-time between evaluations. Breeders could then better select for new parents which could translate into more resilient lines being released for farmers to use.

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CHAPTER 2: DEVELOPMENT OF A HYDROPONIC METHOD TO EFFICIENTLY INDUCE SOYBEAN RESPONSE TO HYPOXIC ENVIRONMENTS

ABSTRACT

Soybean [Glycine max (Merr.)] yields are reduced following hypoxic, waterlogging conditions. The University of Arkansas presently uses a time-restrictive and labor-intensive field screening to identify genotypes for the development of flood-tolerant cultivars. In this research, we tested various germination media and a low-oxygen treatment in order to develop a hydroponic culture method that could be used to screen soybean for responses to hypoxic conditions. First, four germination substrates (rockwool pellets, clay pebbles, perlite, and a hybrid of rockwool pellets in clay pebbles) were evaluated by direct-sowing seeds of three genotypes. Germination percentages were calculated 14 d after planting and analyzed for differences to hydroponic industry standard as the control (rockwool pellets). Secondly, we planted one flood-susceptible soybean genotype in the hydroponic system and subjected it to a hypoxic treatment, displacing O_2 by means of bubbling CO_2 into the system for five days. Plant responses (normalized difference vegetation index (NDVI) and soil-plant analysis development (SPAD)) were evaluated at four intervals following treatment. The germination rate in the control rockwool pellets treatment (58.3%) did not differ ($p=0.586$) from the clay pebbles treatment (58.3%) but was greater than in the perlite ($p=0.008$) (47.9%) and the hybrid media ($p<0.001$) (45.5%). The NDVI and SPAD responses to hypoxic treatment differed ($p<0.001$) from those in the ambient air treatment control (0.292 compared to 0.687 for NDVI, and 10.22 compared to 25.46 for SPAD) across all runs. These results indicate that direct sowing into clay pebble media was an acceptable alternative and time saver as compared to transplanting rockwool pellets into the net pots of the hydroponic systems. Also, the hypoxic treatment created by CO_2 infusion was an effective way to produce leaf yellowing and reduced plant growth.

INTRODUCTION

The global population continues to grow toward a projected total of approximately 9 billion people by 2050, which will require agriculture production trends to increase by 50% to meet projected food demand (Alexandratos & Bruinsma, 2012). There is a need for a substantial increase of yields to meet forecasted demands, but there are many environmental challenges that currently limit crop production (Hunter, Smith, Schipanski, Atwood, & Mortensen, 2017). Developing crops that will be able to meet these demands requires the application of classical, modern, and novel breeding techniques. A key step in all methods of breeding is the identification of valuable traits that can be used to improve existing varieties or develop altogether new cultivars (Richards et al., 2010). Traits that allow a crop to be more robust to biotic and abiotic stressors that would otherwise restrict a high-yielding variety from attaining its full yield potential may even contribute to improved yields under optimal conditions (Beebe, Rao, Blair, & Butare, 2009; Gilliham, Able, & Roy, 2017). Germplasm screening is a critical step in the breeding process that can be improved using existing phenotyping technologies in nontraditional environments (Fiorani & Schurr, 2013; Ghanem, Marrou, & Sinclair, 2015). Through a combination of controlled conditions in a greenhouse, hydroponic systems, and quantifiable observations, it is possible to innovate a more efficient method to isolate traits of interest and thereby increase the rapidity of crop development (Fahlgren, Gehan, & Baxter, 2015).

Field flooding is a leading cause of crop yield losses globally (Lesk, Rowhani, & Ramankutty, 2016). It is estimated that, as global warming accelerates, the weather events that result in abiotic stresses will continue to increase in frequency and intensity (Arnel & Liu, 2001; Rosenzweig, Tubiello, Goldberg, Mills, & Bloomfield, 2002; Mittler & Blumwald, 2010;

Scheirmeir, 2011; Bailey-Serres et al., 2012; Aryal et al., 2019). Given the sensitivity of most crops to waterlogged soils, there is a need to develop crops that are flooding tolerant to minimize yield loss (Manik et al., 2019; Zhang et al., 2019). Plants have been observed to possess adaptations to tolerate short periods of flooding and complete their lifecycles. Adaptations include anatomical features, such as lenticels and aerenchyma, which transport oxygen from above the soil line to oxygen-deprived roots below, as well as physiological adaptations, such as reduced stomatal conductance (Bacanamwo & Purcell, 1999a; Boru et al., 2003; Araki, 2006; Parent, Capelli, Berger, Crèvecoeur, & Dat, 2008; Shimamura, Yamamoto, Nakamura, Shimada, & Komatsu, 2010). Plants also have adapted anaerobic metabolic pathways that allow for adenosine triphosphate (ATP) production under restricted O_2 availability (Parent et al., 2008; Voesenek & Bailey-Serres, 2014).

The use of a greenhouse setting allows for the greater management of uncontrollable environmental factors present in traditional field evaluations, specifically temperature, humidity, and climate (Bennis, Duplaix, Enéa, Haloua, & Youlal, 2008). Abiotic factors, such as water deficit, excessive rainfall, spatial variability, and variable temperatures, make it difficult to generate accurate, reproducible results. Additionally, it is possible that inferences or conclusions based on field evaluations may be confounded with lurking variables, specifically biotic stresses (Bostock, Pye, & Roubtsova, 2014). Control of biological pests is more easily exerted in a greenhouse (Pilkington, Messelink, van Lenteren, & Le Mottee, 2010). Use of a hydroponic system allows plants to develop quickly by providing a constant supply of water, nutrients, and O_2 to the rootzone (Soffer, Burger, & Lieth, 1991; Sardare & Admane, 2013; Palande, Zaheer, & George, 2018). Hydroponics allow for faster growth than traditional, soil-based systems (Gashgari, Alharbi, Mughrbil, Jan, & Glolam, 2018; Wallach, 2019). Furthermore, by combining

the use of a greenhouse setting and a hydroponic system allows for precision and control regarding uniformity of optimized treatments and repeatability of experiments compared to varied field conditions (Kozai, Kubota, & Jeong, 1997).

The objective of these studies was to develop a methodology to expeditiously induce a response that is consistent with soybean symptoms observed in hypoxic, flooded fields that also contain elevated levels of CO_2 (Ponnamperuma, 1984; Kirk, 2004), thus allowing the identification of genotypes sensitive to hypoxic conditions. Two experiments were performed to develop this methodology. In Experiment 1, we evaluated soybean germination rates on four substrate treatments with the objective to identify a substrate that allows direct seeding into hydroponic system, thus increasing efficiency by reducing labor inputs and eliminating seedling transplant shock. In Experiment 2, we grew a soybean cultivar under hypoxic conditions induced by CO_2 infusion, and evaluated plant response using proximal sensing at four predetermined intervals following the gas regimen, comparing plant responses to a control that was grown hydroponically and exposed to ambient air. It was hypothesized that an alternative substrate could be identified for the purpose of direct sowing seeds and that a CO_2 gas treatment could produce significantly different plant responses when compared to an ambient air treatment in hydroponic systems.

MATERIALS AND METHODS

Growing Conditions, Germplasm, and Treatments

All runs (repetitions) of both experiments were conducted in a greenhouse within the Harry R. Rosen Alternative Pest Control Center (RAPC) at the University of Arkansas in Fayetteville, AR. Experiment 1 was conducted twice from December 2018 to January 2019. Experiment 2 was conducted twice from February to April 2019 and again from February to

March 2020. The greenhouse was set to maintain temperatures 20-29° C, using a four-stage cooling system and radiant heating, as necessary. Mean daily average temperature across all experiments was 23°C. Supplemental lighting was set to operate for 13 hours a day (7 a.m. – 8 p.m.) and was set to be on only if solar radiation was below 185 µmol m^{-2} s^{-1}; supplemental lighting provided an average photosynthetic photon flux density (PPFD) intensity of 140 µmol m^{-2} s^{-1} at the base of the plants.

Three independent hydroponic systems were built to develop a protocol to rapidly evaluate response of soybean to a hypoxic environment. The systems were a hybrid of Dutch buckets and recirculating deep-water hydroponics. Forty, 15-L buckets (Affordable Buckets LLC, Victor, IA, United States) were joined by a network of poly vinyl chloride (PVC) pipes and joints, with the terminal ends of the pipes being inserted into lateral grommets near the tops and bottoms of a single side of the buckets. The pipes allowed nutrient solution to be pumped into the lower portion of the bucket from the reservoir and excess nutrient solution to drain from the upper portion of the bucket back into the reservoir. Each forty-bucket system was placed atop one of the three benches in the greenhouse. The origin of circulation of each system was one of three, 600-L stock tanks acting as the reservoir of the system (Rubbermaid Specialty Products Inc., Atlanta, GA, United States), each containing an EcoPlus Eco 1584 submersible pump (Hawthorne Gardening Company, Vancouver, WA, United States) capable of pumping the solution at a rate of 4700 L $hour^{-1}$ through the network of pipes connecting the corresponding system above. A mesh pot, 15.24 cm in diameter by 12.7 cm long, was built into each 15-L bucket and used to support the growth media.

Ambient air was supplied to the nutrient solution with an EcoPlus HGC728459 pneumatic air pump (Hawthorne Gardening Company, Vancouver, WA, United States) that used

a valved, 12-port manifold to supply a similar rate to each bucket. Uniformity of response was validated via the collection of DO data from solution within the pots. Ambient air was supplied at a rate of 5.6 L min^{-1} pot^{-1} (~60 mL min^{-1} pot^{-1} of atmospheric CO_2). Two ports were closed off using the valves; each of the other 10 ports were connected to four buckets using 6.35-mm-diameter, polyethylene vinyl (PEV) air tubing. The tubes terminated in each bucket, connecting to an Air Injection Technology capillary tube (Modular Hydro, Las Vegas, NV, United States). In Experiment 2, the existing air supply system was used to supply CO_2 gas to the hydroponic system by removing the manifold from the air pump and attaching it to a flow meter with an adjustable hose clamp. The flow meter screwed onto the CO_2 gas cylinder valve port, where rate could be adjusted with a dial.

Nutrient Solution

Experiment 1 did not require supplemental fertility, as seed endosperm contains all necessary nutrients and minerals required for the embryo to germinate. Therefore, a modified Hoagland solution No. 1 (Hoagland & Arnon, 1950) was only used in runs of Experiment 2. Stock fertilizer solutions were mixed prior to additions per formula ratios (Supplementary Table S1) then aliquoted and added to each system at the V1 growth stage. The salts used to prepare the fertilizer stock solutions included: calcium nitrate heptahydrate, potassium nitrate, potassium phosphate, magnesium sulfate heptahydrate, boric acid, manganese sulfate hydrate, zinc sulfate heptahydrate, ammonium molybdate tetrahydrate, cupric sulfate pentahydrate, and iron chelate. Soluble components of the solution were: 175 mg N L^{-1}, 54 mg P L^{-1}, 137 mg K L^{-1}, 24 mg Mg L^{-1}, 0.54 mg Mn L^{-1}, 0.49 mg B L^{-1}, 140.24 mg Ca L^{-1}, 0.046 mg Zn L^{-1}, 0.002 mg Mo L^{-1}, 0.025 mg Cu L^{-1}, 1.24 mg Fe L^{-1}, and 32.44 mg S L^{-1}. A combination meter (Bluelab Corporation, Tauranga, New Zealand) was used to monitor electrical conductivity (EC) and pH throughout all

runs of each experiment. The systems were inspected daily, and solution was checked every three days (with exception to daily pH monitoring in run three of Experiment 2).

Sanitization of Hydroponic System and Planting

At the termination of an experiment, gas cylinder valves were turned off and flow meters were removed from the cylinder coupling. If applicable, air pumps were unplugged from power source. Plants were then cut at the soil line and discarded. Hooks, mesh, and bands used to submerge pots into solution were removed and discarded. The clay pebble media was collected and sterilized in an autoclave at 200°C for one hour. Water pumps were unplugged from the power source and drain plugs were removed. Each pot was inspected for residual plant matter or stray clay media, which was removed and discarded. Then, Microbloc concentrated greenhouse disinfectant (alky dimethyl benzyl ammonium chloride 10%, alkyl dimethyl benzyl ammonium chloride 10%) (Floralife, Inc., Walterboro, SC, United States) was diluted with water at a rate of 4 mL L^{-1}, then applied with a pump sprayer to each of the systems. Every surface, including the interior surfaces of pipes for solution plumbing, was sprayed to the point of saturation with the sanitizing solution and allowed to dry. At this point, each system was sanitized and ready to be planted.

Experiment 1: Effects of Direct Sowing into Four Hydroponic Substrates

Four substrates were used to germinate soybean in hydroponic conditions. The first treatment was the industry standard of pre-germination in rock-wool cubes for subsequent transplant once plantlets reached the one-leaf stage. The remaining treatments consisted of direct planting into a hydroponic solution, and substrates included expanded clay media, coarse perlite, and a hybrid method in which the seeds were planted directly into rock-wool cubes that were placed into and covered with expanded clay media. Twenty seeds were planted per bucket, and

there were 12 buckets for each genotype per bench, and three benches per greenhouse. There were three replications of each substrate by genotype combination within each bench, totaling nine replications within each repetition of the experiment. All media in the net pots were topped with approximately 3 cm of the corresponding treatment media (perlite/clay pebbles). Rock-wool controls were not covered, but simply hand-watered when drying of the upper portion of the media was observed. Three commercial soybean genotypes (AG4232, AG46X7, and AG5335) were used, and treatments were arranged based on a randomized complete block (RCB). Germination rates were determined by counting seedlings 10 d after planting. At this time, plants were also measured using a handheld Greenseeker normalized difference vegetation index (NDVI) sensor placed 50 cm above the pot (Trimble Inc., Sunnyvale, CA, United States) to estimate seedling vigor.

Experiment 2: Hypoxia Response of a Flood-sensitive Soybean Cultivar

The variety UA5014C, a maturity group (MG) 5 cultivar, was selected to assess hypoxic treatment because this cultivar has been previously screened under flooded field conditions and was shown to be consistently flood-sensitive at multiple growth stages over the course of five years (Wu et al., 2017a; Wu et al., 2017b; Wu et al., 2017c). For this experiment, seeds were directly planted in clay media as identified in Experiment 1 and grown to the V2 growth stage (Fehr and Caviness, 1977). At that point, the mesh pots of both the control and treated systems were lowered into the solution and clamped down, and plants were submerged up to the unifoliate leaves by manually pushing stems down into the media. This was done to better identify other traits associated with flood tolerance, as opposed to allowing plants to develop aerenchyma (Carlin, 2014; Linkemer et al., 1998; Maekawa et al., 2011; Hummer, 2018).

Following submersion, air pumps were shut off for the treatment bench and left on for the ambient air treatment bench. By leaving the pumps on for the ambient air treatment bench, plants could continue to grow under optimal non-stress conditions as a control. The air supply manifold was then removed from the pump and attached to the CO_2 gas cylinders, followed by initiation of CO_2 treatment at a rate of 200 mL min^{-1} $plot^{-1}$. Treatment was applied, uninterrupted, for five consecutive days. Dissolved oxygen (DO) of the solution was measured using a handheld DO meter (Model HI9142, Hanna Instruments, Woonsocket, RI, United States) in both the treatment and control systems. A DO value of 5 mg L^{-1} was previously reported to be the critical threshold at which detrimental effects occur in plants (Drew, 1996). Dissolved oxygen was monitored to ensure the treatment solution fell below the critical value to a range consistent with flooded fields to validate susceptibility of the experimental genotype.

In addition to the drop of pH in the treatment system due to the natural formation of carbonic acid (H_2CO_3) when CO_2 is dissolved in water (Hirshberg & Gerber, 2016), the pH of the Hoagland solution tends to rise over time (Wang, Fu, & Liu, 2016), creating a greater difference between the two pH levels. Therefore, in run three of Experiment 2, pH of the nutrient solution was adjusted as needed in the control system. The pH of the control bench nutrient solution was measured twice daily, using a pH meter, and anhydrous, food-grade citric acid ($C_6H_8O_7$) (Naturevibe Botanicals, Rahway, NJ, United States) was mixed with deionized water to a strength of 1M and added to the control bench, twice daily, to adjust pH value of the nutrient solution to be similar to that of the treatment bench.

Plant responses for both the hypoxic and control treatments were evaluated using NDVI (Greenseeker) and SPAD (Konica-Minolta SPAD-502) meters at the day of termination of gas treatment (day 0), as well as for the following 3, 6 and 9 days after. The NDVI values were

measured at a height of approximately 50 cm above the top of plants in each plot, and SPAD values were taken from the lateral leaflet of the uppermost, fully unfurled leaf 1 for each plant in a plot and later averaged across plants to create a mean plot SPAD response. The SPAD and NDVI indices were selected to evaluate tolerance and susceptibility because they measure plant greenness as a function of total chlorophyll content. These indices have been previously used to evaluate water stress responses in soybean (Mokua, 2015; Xiong et al., 2015; Lee, Jung, Chun, & Choi, 2017; Hummer, 2018), of which a common, early indicator of damages is chlorosis (Sullivan et al., 2001; Nguyen et al., 2012).

Analysis

Experiment 1 was analyzed using SAS 9.4 statistical software (SAS Institute Inc., Cary, NC, USA). A generalized linear mixed model (GLIMMIX) procedure was used to conduct analysis of variance (ANOVA) to evaluate the effects of substrate treatments on germination rate and NDVI response. A multiple comparison test for seed germination percentage across substrates was performed using the Tukey HSD with a significance level $\alpha=0.05$.

The following model was used for all Experiment 1 analyses:

$$Y_{ijkl} = \mu + Substrate_i + Variety_j + Substrate*Variety_{ij} + Run_k + Rep(Bench)_l + Bench(Run)_m + \varepsilon_{ijklm}$$

Where $Substrate_i$ is the fixed effect for the number of substrate media treatments, $Variety_j$ is the fixed effect for the number of genotypes, $Substrate*Variety_{ij}$ is the fixed effect for the interaction between the substrates and genotypes used, Run_k is the random effect for each repetition of the experiment, $Rep(Bench)_l$ is the random effect for replications nested within bench, and

$Bench(Run)_m$ is each bench (block) nested within run. The assumed distribution was based on $Run_k \sim N(0, \sigma^2)$.

Experiment 2 data were also analyzed using SAS 9.4 statistical software as well as JMP Pro 14.2 statistical software (SAS Institute Inc., Cary, NC, USA). A generalized linear mixed model (GLMMIX) procedure was used to evaluate differences between treatments (i.e., hypoxia, control), days after treatment (DAT) and their interaction on germination and NDVI response. Fitted Least Square models were used to calculate significant differences of solution responses between the two treatments as well as the effect of plant responses.

The following model was used for the ANOVA of experiment 2:

$$Y_{ijkl} = \mu + Treatment_i + DAT_j + Treatment*DAT_{ij} + Run_k + \varepsilon_{ijk}$$

Where $Treatment_i$ is the fixed effects for the CO_2 and ambient air treatments, DAT_j is the fixed effect for the days after treatment interval at which responses were recorded, $Treatment*DAT_{ij}$ is the fixed effect for the interaction between the treatments and the day after treatment observation interval, and Run_k is the random effect for repetition of the experiments. For NDVI and SPAD, an ANOVA was performed using the GLIMMIX procedure in SAS assuming a Beta distribution for NDVI and a gamma distribution for SPAD.

RESULTS

Experiment 1

Germination percentage differed among substrates ($p=0.0004$) and differed among genotypes ($p<0.001$) effect, but no significant genotype-by-substrate interaction ($p=0.1500$) (Table 1). It was observed that the standard treatment control using rockwool pellets (58.3%) differed from the perlite treatment (47.9 %) ($p=0.008$) and the hybrid treatment (45.4%)

($p<0.001$) did not significantly differ from the clay pebble treatment (54.3%) ($p=0.586$); clay pebbles also did not differ from the perlite treatment ($p=0.200$) (Table 2). Additionally, germination rates varied by genotype (Table 3), likely due to varied seed source. For the purposes of the methodology being developed, clay pebble media was concluded to be an acceptable alternative to the standard rockwool cubes for germination of seeds to be grown in a hydroponic system regardless of the genotype and seed quality used.

Similar to germination rate, NDVI differed among genotypes and substrates effects ($p<0.001$, $p<0.001$, respectively), but non-significant interactions between the two (Table 4). Perlite NDVI (0.274) differed from clay pebbles (0.398) ($p<0.001$), the hybrid treatment (0.372) ($p<0.001$), and rockwool cubes (0.362) ($p=0.003$), which did not differ among one another (Table 5).

Experiment 2

The effects of the CO_2-induced hypoxia treatment on the properties of the nutrient solution and plant responses, including pH, DO, EC, SPAD and NDVI, are summarized below. Responses for all factors were dynamic with timing of observations except for EC. Despite the variable responses of SPAD, the mean SPAD responses did not differ significantly between the hypoxic treatment and the ambient air treatment for all observed intervals.

Nutrient Solution

Hypoxic CO_2 treatment and ambient air treatment pH levels differed at measurement intervals of 0 DAT ($p=0.0005$) (pH 5.26 and 6.11, respectively), 6 DAT ($p=0.0004$) (pH 6.75 and 7.28, respectively), and 9 DAT ($p=0.0067$) (pH 6.86 and 7.69, respectively), but not 3 DAT ($p=0.2312$) (pH 6.92 and 6.84, respectively) (Figure 1a). For the hypoxic CO_2 treatment and the ambient air treatment, pH levels differed ($p=0.0067$) from one another when averaged across all

intervals and runs (6.45 and 6.98, respectively). The treated and untreated system solutions' pH increased with the passing of time beginning at 0 DAT; however, it is worth noting that pH was outside of the optimal ranges for soybean at 0 DAT (CO_2 treatment only, 5.26) and 9 DAT (ambient air treatment only, 7.69). Dissolved oxygen was above the critical level of 5 mg L^{-1} for all DAT intervals except for 0 DAT (CO_2 treatment) and levels differed ($p=0.0016$) between the CO_2 treatment (4.68 mg L^{-1}) and ambient air treatment (6.21 mg L^{-1}) across all observation intervals (Figure 1b). Dissolved salts in the two systems did not differ at any of the observation intervals and averaged 1.57 to 1.60 dS m^{-1} throughout (Figure 1c).

NDVI

The level of greenness for UA5014C plants as a function of NDVI was measured at four DAT intervals. The average NDVI responses were 0.292 and 0.687 for the CO_2 treatment and ambient air treatment (control), respectively. The NDVI response differed between treatments over time ($p<0.001$), indicating that responses were dependent on the timing of data collection due to interaction between the two factors ($p<0.001$) (Table 6). At 9 DAT, the greatest difference in NDVI response occurred between the hypoxic CO_2 treatment (6.91) and the ambient air control (25.13). This difference was marginally higher for 9 DAT than 6 DAT (0.13), though not statistically different (Table 7). The identification of the interval with the greatest separation of response is relevant to the focus of improving time efficiency of screening. There was a decrease of NDVI response in the treated system as time progressed beyond the termination of treatment, whereas the control bench maintained a steady result for both responses. There were differences in the ordered least square means ranking of DAT intervals for the NDVI response, where the ambient air treatment responses differed from the CO_2 treatment responses for all DAT intervals (Table 7).

SPAD

The change in chlorophyll response of UA5014C plants as a time series was measured as a function of SPAD units following a five-day hypoxia treatment created with CO_2 gas additions. The average SPAD response was 10.22 and 25.46 in hypoxic and ambient air systems, respectively. The SPAD response differed between treatments over time (significant interaction effect) ($p<0.001$), also indicating that responses were dependent on the timing of data collection, as occurred for NDVI. Interval DAT 3 had the greatest difference (contrast of -18.36) between SPAD responses for the CO_2 treatment and the ambient air control. Responses for SPAD were greater (15.24) for the ambient air treatment compared to the CO_2 treatment at all time intervals, while SPAD did not differ among any time intervals for the ambient air treatments on any of the measurement days for the CO_2 treatment (Table 8).

DISCUSSION

Experiment 1

Direct sowing reduces cycle time by removing the unnecessary step of germinating seeds in one media/location and transferring to a final media/location. Presently, rockwool cubes are the primary substrate used for seed-starting and soilless culture in commercial applications due to achievement of large yields (Jeong & Hwang, 2000; Allaire, Caron, Ménard, & Dorais, 2005). However, the horticultural industry standard for greenhouse and hydroponically grown, seed-started vegetable crops relies on germination in rockwool cubes prior to transplantation into hydroponic systems or rockwool slabs (Bussell & McKennie, 2004). Additionally, prior studies exploring hypoxia specified the application of procedures that used pre-germination and transplantation into hydroponic systems (Boru et al., 2003; Araki, 2006; Jitsuyama, 2015). Of the germination media evaluated, the coarse perlite was the least conducive to applications of this

methodology to evaluate germplasm responses to hypoxic conditions, despite being a standard media for vegetable production (Tyson, Hochmuth, Lamb, Hochmuth, & Sweat, 2001). The aggregate size of the perlite substrate required mesh liners in the net pots to prevent the loss of media, increasing labor by an estimated 10% and materials costs by an estimated $0.20 per pot for each repetition, which are in direct contrast to the objectives of this experiment and is therefore not recommended as an alternative direct-seeded, germination media for deep-water hydroponic culture.

The use of clay-pebble media provides advantages over other hydroponic growing and germination media. Specifically, there is an economic advantage in that clay-pebbles are reusable since they can withstand the high-temperature sterilizing conditions of an autoclave; the large particle size of the pebbles also allows for ease of cleaning with water. The recyclable nature makes the pebbles a sustainable alternative to rockwool cubes as well and could possibly even meet the need for a renewable, environmentally sound alternative as a soilless substrate in the horticulture industry as sought by Allaire et al. (2005). Additionally, the ability to direct-sow seeds eliminates the need for pre-germination and transplantation of seeds and seedlings, thereby reducing labor costs and further increasing economic efficiency.

The use of the clay-pebble substrate was only explored using a recirculating, deep-water hydroponic system. Despite the clay-pebble media having been established as suitable for hydroponic vegetable production following transplantation (Szilágyi, Slezák, Ferenczy, & Terbe, 2006), use of clay-pebbles as an alternative to pre-germination in rockwool cubes may be restricted to recirculating, deep-water hydroponic systems. Also, because of the texture of the material, it is necessary that the clay-pebbles are autoclaved or sterilized to make them viably reusable.

The economic and sustainable factors of the clay-pebbles make the substrate a suitable candidate to be implemented in a screening methodology that is efficiency oriented. Further investigations to test the effectiveness of this media as an alternative germination media to rockwool cubes in other types of hydroponic systems are still required. The same clay-pebble media was first used in these experiments in the spring of 2019 and have currently been re-used for a total of eight experiments; where the period of use was mostly consecutive. There is an opportunity to establish the lifetime of the medium and determine how long the media can be recycled before breaking down to further validate economic superiority of clay-pebbles over other hydroponic media currently used in greenhouse industries.

Experiment 2

Soybean plants growing in waterlogged fields display foliar symptoms in response to anoxic, elevated-CO_2 conditions that stress the plant (Ponnamperuma, 1984; Kirk, 2004); thus, the creation of similar conditions in a controlled environment was expected to produce similar responses. Hypoxic conditions were successfully created by displacing dissolved O_2 via infusion of CO_2 into the nutrient solution of a hydroponic system, as evidenced by the measured DO differences between the treatment and control systems. The following conclusions are predicated upon the established premise that significantly differing DO concentrations were present in the two systems at the same intervals for all runs of Experiment 2; one hypoxic (oxygen-deficient, below the critical threshold 5.0 mg L^{-1}) and one sufficiently oxygenated (above the critical threshold 5.0 mg L^{-1}). There were significant differences between plant response (NDVI or SPAD) of the single genotype, UA5014C grown under hypoxic conditions created by a CO_2-gas treatment compared to the ambient air treatment. This result suggests that the hypoxic, CO_2

treatment would be effective to aid in a high-throughput method to evaluate varied germplasm response to low-O_2 environments.

While there were significant differences between the CO_2 treatment and the ambient air treatment across the whole experiment for NDVI and SPAD responses, there were also significant differences between the DAT intervals for the responses evaluated within the CO_2 treatment and the ambient air treatment. It is important to isolate the interval with the greatest level of separation between these treatments with the purpose of potentially applying the method to a screening protocol. The interval in which the largest separation occurs would theoretically be the optimal point to evaluate diverse germplasm for tolerance and susceptibility. Previously, controlled-environment studies have merely explored the required intervals to induce response and the corresponding level of severity, with no concentration on maximizing the differences between the ambient air treatment and the hypoxic, CO_2-treated varieties. Furthermore, isolating a single evaluation reduces the labor costs associated with repeated measures. A second factor contributing to optimization to consider is the sensing technique that provides the widest separation between the treatment and the control. The NDVI data had lower variance than the SPAD data and has long been used for identification of abiotic stress in crops (Watt et al., 2020). Also, the NDVI meter can measure whole plots, whereas the SPAD meter requires the measurement of each plant in a plot to calculate a SPAD average, which may contribute to the variance of responses.

In contrast to results from Boru et al. (2003), there were significant changes in solution pH following CO_2 additions. However, the changes became less significant by 3 DAT. In the third run of the hypoxia experiment, pH was adjusted in the ambient air treatment control bench. The change in pH had no significant bearing on the outcome of plant responses between the

ambient air and CO_2 treatments compared to other runs. As pH increased following treatment, so did the DO levels in the CO_2-treated bench, meanwhile there was a steady downward trend for NDVI and SPAD responses, suggesting that plants remain stressed even as pH and DO approached levels consistent with the ambient air treatment bench. The level of observed variability attributed to random error caused by the Run factor was very low for both NDVI and SPAD responses, indicating that the techniques explored in these experiments were reliable and could repeatably produce consistent results.

Prior research focusing on soybean response to waterlogging and hypoxia stress has primarily been conducted at the early reproductive stage as this stage has been identified as the point at which the crop is most sensitive to yield losses. However, by screening for early vegetative tolerance, it is possible to create varieties that can overcome the stresses of early season flooding that often occurs in areas prone to flooding. The creation of cultivars tolerant to early vegetative hypoxia stress offers an economic advantage to producers in the form of improved stands that translate to greater yields and improved food security. Furthermore, adapted cultivars may confer yield advantages under optimal growing conditions (Beebe et al., 2009; Gilliham et al., 2017). Similar previous studies have focused on the mechanisms and pathways by which hypoxic stresses affected plants, without emphasizing the potential of using observed tolerance in a breeding application (Boru et al., 2003; Araki, 2006; Hossain & Uddin, 2011; Duhan, Kumari, Lal, & Sheokand, 2019; Bashar, Tareq, & Islam, 2020). A subsequent goal of this research was to apply this method to future germplasm screenings similar to the field studies conducted by Wu et al. (2017b; 2017c). An added advantage of the greenhouse to meet goals is that the greenhouse allows for the stable recreation of early season environmental factors, such as temperature and photoperiod, observed in fields where potential cultivars would

be grown, aiding in the selection of parental lines that exhibit the ability to withstand low-oxygen environments that can be crossed with high-yielding germplasm.

One of the greatest limitations of this experiment was that exact levels of dissolved CO_2 were not measured. Therefore, the critical, supra-optimal value at which damages are incurred from CO_2, opposed to simply the lack of oxygen, may not be inferred. Bacanamwo & Purcell (1999), as well as Boru et al. (2003), effectively demonstrated that anoxia alone was not sufficient to induce symptoms of waterlogging stress. Additionally, the rates of CO_2 additions for this experiment were chosen as a best estimate based on contrasting rates used in previous studies measuring plant and gas interactions in the rhizosphere (Boru et al., 2003; Araki, 2006; Jitsuyama, 2017). Also, previous studies would sometimes use carrier gases, such as helium or nitrogen, causing difficulty to estimate the best rate, especially given the range of observed chlorosis and plant survival rate responses in those studies. Boru et al. (2003) provided the best estimate for use of 100% CO_2 gas at a rate of 300 mL min^{-1} to observe plant death of susceptible cultivars.

The ability to evaluate roots in a nondestructive manner due to lack of soil coupled with the large media size of the clay pebbles, especially when compared to field evaluations, offers an opportunity to further explore how root morphology correlates to tolerance and susceptibility in soybean. To maximize efficiency and optimize plant phenotyping, based on our findings, use of NDVI sensing at 6 DAT at treatment would likely provide the most accurate results in a germplasm screening trial that is evaluating tolerance of soybean plants grown under CO_2-induced hypoxic conditions in a hydroponic system. Ideally, 0 DAT would best maximize time efficiency, but 0 DAT must be ruled out due to plants possibly interacting with pH ranges marginally outside (5.26) of the optimal soil pH range (6.0-6.8) for soybean. Additionally, a pH

of 5.26 is not truly below hydroponics optimum value, it is however close to the lower limit of pH 5. Certainly, the occurrence of pH values outside the optimal range could occur, as the 5.26 value is only an average, or are close enough that this interval should not be considered. The optimal rate of CO_2 additions could be better identified with gas chromatography (Boru et al., 2003; Araki, 2006), an optimized level of CO_2 holds the potential to limit pH interactions and could increase the efficiency of this protocol.

CONCLUSIONS

Ranked correlation of germination and NDVI responses of three commercial soybean (AG4232, AG46X7, and AG5335) varieties to the tested substrates, determined from evaluations in the greenhouse, indicated that direct sowing into clay pebbles was comparably effective as the use of rockwool cubes, which potentially increases time efficiency and reduces labor inputs. Results from plant responses of a flood-susceptible soybean cultivar (UA5014C) following a period of hypoxia in a hydroponic system demonstrated that greenhouse screening could be a useful technique to evaluate hypoxia tolerance in soybean in early vegetative growth stages, but the timing of evaluations is critical to effectively parse out the responses. Furthermore, based on contrasting plant responses in the control and treated systems, results indicated that 6 DAT is the optimal interval of evaluation for both NDVI and SPAD when factoring the possibility of pH interactions. The compound use of these results, as well as the short cycle time of five weeks, offers the opportunity to expedite the screening protocol compared to current field evaluations; time- and cost-efficiency would be improved, especially when factoring the potential to conduct year-round screenings. The cost of a single flood-trial research plot for the University of Arkansas Soybean Breeding Program is approximately $15.00 including labor, the cost of a plot using the methods outlined herein are approximately $13.00 per plot, not including labor or the

initial investment of hydroponic systems. The inclusion of these results into a high-throughput screening method could help overcome the bottleneck of identifying suitable parents that confer waterlogging tolerance traits to an adapted cultivar to be grown in agronomically important, flood-prone regions.

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TABLES AND FIGURES

Table 1. Covariance parameter estimates and Type-III tests of fixed effects for ANOVA of germination rate percentage response as function of substrate and variety.

Covariance Parameter Estimates

Cov Parm	Estimate	Standard Error
Run	0	.
Rep(Bench)	0.005581	0.009861
Bench(Run)	0.1638	0.1111
Residual	0.02630	0.002678

Type III Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Variety	2	193	127.55	<0.0001
Substrate	3	193	6.39	0.0004
Variety*Substrate	6	193	1.56	0.1500

Table 2. Tukey honest significant difference (HSD) substrate ranking based on germination percentage.

Substrate	Germination %			
Rockwool Pellets	58.3	A		
Clay	54.3	A	B	
Perlite	47.9		B	C
Rockwool/Clay Hybrid	45.4			C

Substrates not connected by the same letter are significantly different at p=0.05.

Table 3. Tukey HSD variety ranking based on germination percentage.

Level	Germination %		
AG4232	79.4	A	
AG46X7	52.5		B
AG5335	22.4		C

Varieties not connected by the same letter are significantly different at p=0.05.

Table 4. ANOVA for normalized difference vegetation index (NDVI) response as function of substrate and variety: Experiment 1, α=0.05.

Effect	Num DF	Den DF	F Value	Pr > F
Variety	2	193	119.66	<0.0001
Substrate	3	193	6.26	0.0004
Variety*Substrate	6	193	1.26	0.2774

Table 5. Tukey HSD substrate ranking based on NDVI response.

Substrate	Mean NDVI		
Clay	0.398	A	
Rockwool/Clay Hybrid	0.372	A	
Rockwool Pellets	0.362	A	
Perlite	0.274		B

Substrates not connected by the same letter are significantly different at p=0.05.

Table 6. Experiment 2 ANOVA output for NDVI and SPAD responses.

Type III Tests of Fixed Effects - NDVI				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	1	950	1602.66	<0.0001
DAT	3	950	3.15	0.0242
Treatment*DAT	3	950	13.17	<0.0001
Type III Tests of Fixed Effects - SPAD				
Effect	Num DF	Den DF	F Value	Pr > F
Treatment	1	946	534.14	<0.0001
DAT	3	946	9.01	<0.0001
Treatment*DAT	3	946	7.63	<0.0001

Level of significance, α=0.05.

Table 7. Ranked responses of NDVI separated by treatment and DAT – Hypoxia Test.

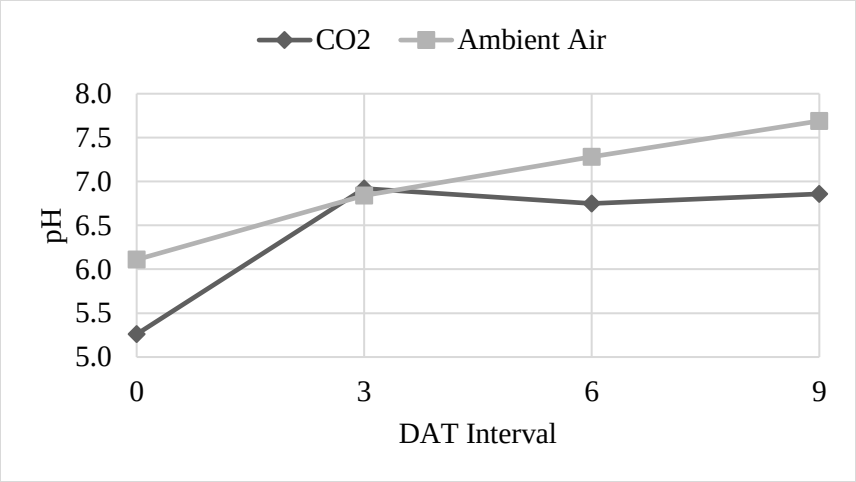
Treatment	DAT	Mean NDVI					
Control	6	0.728	A				
Control	9	0.712	A	B			
Control	3	0.671		B	C		
Control	0	0.637			C		
CO_2	0	0.342				D	
CO_2	6	0.321				D	E
CO_2	9	0.287					E
CO_2	3	0.219					F

Means not connected by the same letter are significantly different, p=0.05. Control is the ambient air treatment.

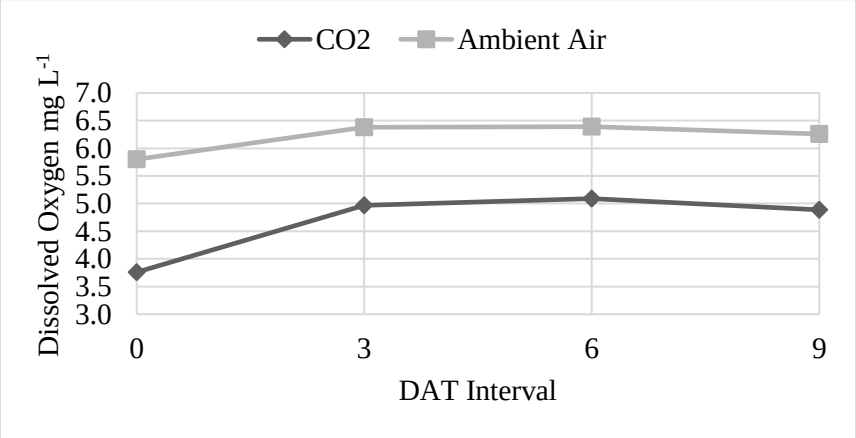
Table 8. T Grouping for DAT*Interaction – SPAD. Least Square Means.

Treatment	DAT	Estimate				
Control	0	30.7	A			
Control	3	30.7	A			
Control	9	28.9	A			
Control	6	28.4	A			
CO2	0	10.8		B		
CO2	9	7.7			C	
CO2	6	6.4			C	
CO2	3	4.6				D

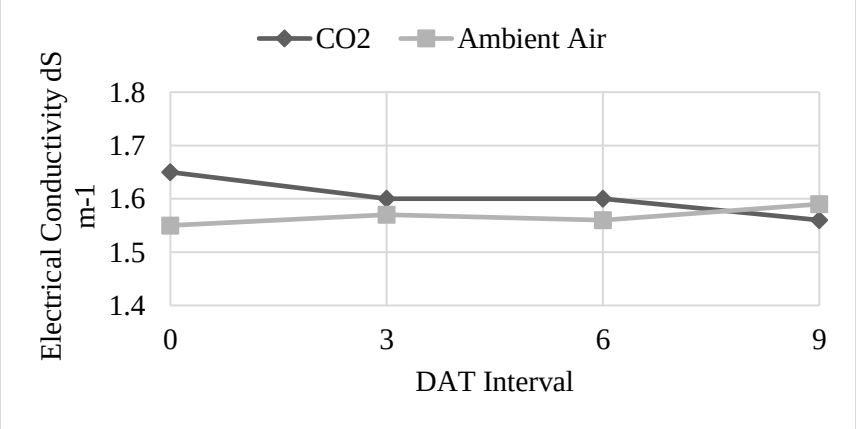
LS-means not connected by the same letter are significantly different, p=0.05.



(A) pH changes over time according to treatment



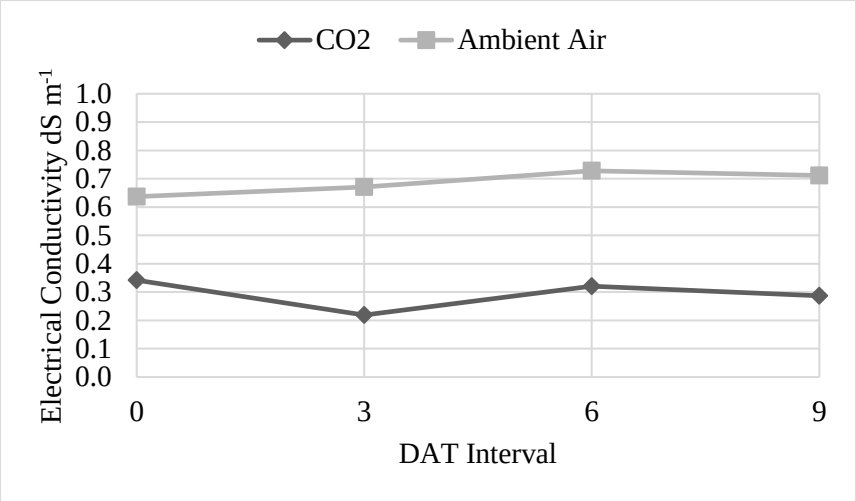
(B) DO changes over time according to treatment



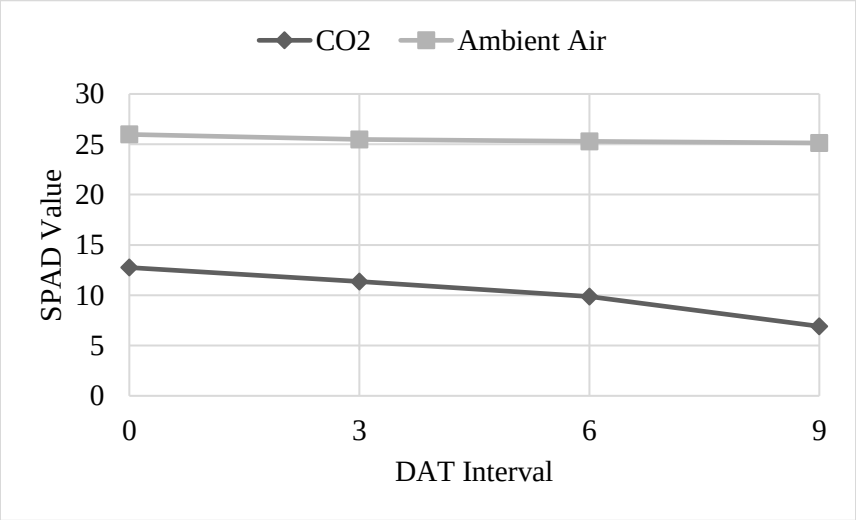
(C) EC changes over time according to treatment

Figure 1. Time series of plant and solution responses at all days after treatment (DAT) intervals. (A) pH (B) dissolved oxygen (C) electrical conductivity (D) NDVI and (E) SPAD.

Figure 1 continued



(D) NDVI changes over time according to treatment



(E) SPAD changes over time according to treatment

Figure 1. Time series of plant and solution responses at all days after treatment intervals. (A) pH (B) dissolved oxygen (C) electrical conductivity (D) NDVI and (E) SPAD

Figure 2. Severity of symptom development of UA5014C when subjected to 5 days of O_2 displacement in a hydroponic environment. Picture taken 9 days after termination of hypoxia. Left image shows plants receiving CO_2 in a hydroponic system, right image shows plant receiving ambient air in a hydroponic system.
Photos by Author.

CHAPTER 3: QUANTIFYING RESPONSE OF VARIOUS SOYBEAN ACCESSIONS TO HYPOXIA IN A HYDROPONIC ENVIRONMENT

ABSTRACT

Soybean [*Glycine max* (L.) Merr.] is a flood-sensitive crop often grown in flood-prone areas. Yield losses can be mitigated by incorporating early vegetative flood-tolerance traits; however, there is a need to identify germplasm with such tolerance. The objective of this study was to test the efficacy of a greenhouse screening method to identify varieties that are tolerant to CO_2-induced hypoxic conditions during early vegetative growth stages. Thirty-three diverse genotypes were planted as a split-split plot design with three replications where the main plot was run (four repetitions from June - December 2019), the sub-plot was genotype, and the sub-subplot was days after treatment (DAT); (0, 3, 6, and 9 DAT). All genotypes were grown in a recirculating hydroponic system and were subjected to hypoxic conditions at the V2 growth stage. Hypoxia was induced by continuous CO_2 additions at a rate of 200 mL min^{-1} for five days. Plant responses were evaluated using a handheld normalized difference vegetation index (NDVI) and soil-plant analysis development (SPAD) sensors, as well as a nine-point visual foliar damage score (FDS). The NDVI index was shown to be the most effective for differentiating germplasm irrespective of the season in which evaluations were conducted, as the variance estimate for run was 0.4% of total variance and the experiment had 94.5% repeatability. The NDVI responses differed among genotypes ($p=0.0002$) and differed over time ($p<0.0001$), there was no significant interaction effect ($p=0.9948$). Significant differences among genotypes indicated soybean possesses varying levels of tolerance to hypoxia. Among genotypes with increased tolerance include PI 471938, R11-6870, and ‘Walters’. Results of this study support the premise that a breeding program could consistently evaluate germplasm under hypoxic conditions year-round to aid in the rapid development of flood-tolerant cultivars.

Abbreviations: ANOVA, analysis of variance DAT, days after treatment; DO, dissolved oxygen; EC, electrical conductivity; FDS, Foliar Damage Score; HSD, honest significant difference; MG, maturity group; MT, moderately tolerant; NDVI, Normalized Difference Vegetation Index; S, susceptible; SPAD, Soil-Plant Analysis Development; T, tolerant

INTRODUCTION

Flood is a prominent abiotic stress and is a major cause of global agricultural yield loss annually (Normile, 2008; Chen et al., 2016; Ye et al., 2018). Soybean [Glycine max (L.) Merr.)] is a good candidate crop to improve flood tolerance as soybean is generally flood-sensitive (Scott, DeAngulo, Daniels, & Wood, 1989; Bacanamwo & Purcell, 1999a, 1999b) and of large economic value (Xing, Popp, Chen, Manjarrez-Sandoval, & Gbur, 2018). Soybean is the second most planted crop in the United States and is an important plant-derived protein source for animal feed globally (Erdaw, Bhuiyan, & Iji., 2016; FAO, 2020). Additionally, soybean is an important grain crop that serves as a major oil source for human consumption (Wilcox, 2004; Norman, 2012). Soybean production was valued at $31 billion in the United States in 2019 (USDA NASS, 2020), which is less than the full potential if flood-tolerant cultivars were planted in flood-prone regions.

Flood-sensitive cultivars of soybean have been observed to incur 20 to 39% lower yields if compared to a flood-tolerant cultivar following waterlogging stress in the Midsouthern U.S. (Rhine, Stevens, Shannon, Wrather, & Sleper, 2010). Previous studies have elucidated mechanisms of flood stress and plant response in soybean (Ahmed et al., 2012; Board, 2008; Jitsuyama, 2015; Mutava et al., 2015). Low O_2 coupled with elevated CO_2 in the rhizosphere reduce nutrient and water uptake and overall biomass accumulation in soybean plants (Boru, Vantoai, Alves, Hua, & Knee, 2003; Araki, 2006). Morphological and physiological adaptations, including adventitious root growth and reduced stomatal conductance, allow soybean plants to complete their lifecycle following flood stress (Thomas, Guerreiro, & Sodek, 2005; Mutava et al., 2015; Coutinho et al., 2018). Of the cultural practices to mitigate flood risk, flood-tolerant

cultivar selection is preferable due to being simple and economical (Henshaw, Gilbert, Scholberg, & Sinclair, 2007; Wu et al., 2020).

Efforts to breed flood-tolerant varieties are ongoing (Wu et al., 2017a; Dhungana et al., 2019), but progress is slowed due to limited availability of accessions with stable traits for flood tolerance. In the past, large amounts of germplasm were screened for tolerance in flooded fields, but the process is laborious and limited to a single observation per year (Mokua, 2015; Wu et al., 2017b). A simplified method to reduce time and labor inputs could accelerate development of flood tolerant varieties.

The purpose of this study was to test the application of methods to screen soybean germplasm year-round quickly and consistently in a greenhouse to identify entries possessing traits for hypoxia tolerance in the early vegetative growth stages. It is hypothesized that when a diverse group of soybean germplasm are subjected to a CO_2-induced hypoxic treatment, plant responses will be varied and can be categorized into classes of tolerance and susceptibility.

MATERIALS AND METHODS

Genotypes

Thirty-three genotypes were used in this experiment (Supplementary Table S2) representing maturity group (MG) 4-7 from diverse pedigrees. Wu et al. (2017a; 2017b; 2017c) previously screened all materials in a flooded-field setting and rated for tolerance at the V5 and R1 growth stages (Fehr & Caviness, 1977). Based on previous ratings, the panel was comprised of 19 tolerant, 2 mildly tolerant, and 11 susceptible genotypes.

Greenhouse and Growing conditions

Four experiments were carried out in a greenhouse in the Harry Rosen Alternative Pest Control Center at the University of Arkansas Campus in Fayetteville from June to December 2019. During experiments, air temperatures of 25 to 29°C were maintained with a photoperiod of 13 h. All 33 genotypes were directly sown with 10 seeds per pot into a clay substrate (Hydrofarm, Petaluma, CA, United States) in a randomized complete block design in three hydroponic systems (block) with one replication each per block for each run, totaling three replications per run. At V1, seedlings were thinned to three plants per pot. The hydroponic system was a deep-water-culture recirculating design, that used an EcoPlus HGC728459 pneumatic air pump (Hawthorne Gardening Company, Vancouver, WA, United States) to infuse ambient air into the nutrient solution under normal growing conditions. At V1, stock solutions of liquid fertilizer, prepared using the Hoagland formulation No. 1 (Hoagland and Arnon, 1950), were added to the hydroponic system solution (unamended water) at a rate of 1mL L^{-1}. The fertilizer stock solutions included the following salts: ammonium molybdate tetrahydrate, boric acid, calcium nitrate heptahydrate, cupric sulfate pentahydrate, iron chelate, magnesium sulfate heptahydrate, manganese sulfate hydrate, potassium nitrate, potassium phosphate, and zinc sulfate heptahydrate. Also, the solution included the following soluble components: 175 mg N L^{-1}, 137 mg K L^{-1}, 140.24 mg Ca L^{-1}, 54 mg P L^{-1}, 32.44 mg S L^{-1}, 24 mg Mg L^{-1}, 1.24 mg Fe L^{-1},0.54 mg Mn L^{-1}, 0.49 mg B L^{-1}, 0.046 mg Zn L^{-1}, 0.025 mg Cu L^{-1}, and 0.002 mg Mo L^{-1}. As described in Chapter 2 of this thesis, plants were grown in the systems until the V2 growth stage, at which point the growing media was secured with a mesh fabric and rubber bands. Subsequently, pots were submerged to cover the lower portion of plant stems and infusion of gas into the nutrient solution was initiated.

Gas Treatment and Response

To induce a hypoxic response, CO_2 was bubbled into the hydroponic nutrient solution of each pot at a rate of 8 L min^{-1} $system^{-1}$ for a duration of 5 d by connecting the pre-existing air supply manifold to a CO_2 gas flow meter attached to a CO_2 source (22.7 kg gas cylinder). Application of the CO_2 treatment was continuous for the 5-d period, apart from switching to full gas cylinders every two days. An ambient air treatment was not used as a control because the CO_2 treatment alone was found to successfully and consistently induce plant responses (Chapter 2).

Upon termination of treatment on day five, solution and plants were evaluated. Dissolved oxygen (DO) in the hydroponic solution was measured using a handheld DO meter (Model HI9142, Hanna Instruments, Woonsocket, RI, United States), and pH and electrical conductivity (EC) were measured using a combination meter (Bluelab Corporation, Tauranga, New Zealand). Foliar damage scores were evaluated using a nine-point visual scale (1 to 9), where 1 represents plants that display not discernable stress or damages and 9 represents plants that are completely dead (Wu et al., 2017c). Plot greenness was measured using a handheld Greenseeker NDVI sensor (Trimble Inc., Sunnyvale, CA, United States). Individual plants within a plot were evaluated for chlorophyll content using a SPAD-502 handheld meter (Konica-Minolta, Tokyo, Japan) by measuring the uppermost fully-expanded lateral leaflet on all three plants in a given plot; an average SPAD value of all plants in a plot was generated for statistical analysis. Successive measurements of hydroponic solutions and plants were repeated at 3, 6, 9 d after treatment ceased.

Statistical Analysis

Analysis of variance (ANOVA) for NDVI, SPAD, and FDS were independently analyzed using SAS 9.4 statistical software suite (SAS Institute Inc., Cary, NC, USA). All models were generalized linear mixed models (GLIMMIX). Experiments were conducted as a split-split-plot design with three replications, each replication planted in a RCBD. Run was the whole-plot factor with four levels, genotype was the sub-plot factor with three levels (bench), and DAT were the sub-sub-plot factor. There were two factors: genotype and DAT. Genotype had 33 levels and DAT had four levels.

The following model was used for all analyses of variance:

$$Y_{ijkl} = \mu + Genotype_i + DAT_j + Run_k + Bench(Run)_l + [Bench*Genotype(Run)]_{ikl} + (Genotype*DAT)_{ij} + \varepsilon_{ijkl}$$

Where $Genotype_i$ was the fixed effect for the number of genotypes, DAT_j was the fixed effect for the number of observation intervals, Run_k was the random effect for number of times the experiment was repeated, and $Bench(Run)_l$ was the number of benches nested within each run. The assumed distribution was based on $Run_k \sim N(0, \sigma^2)$.

The following equation was used to calculate repeatability for NDVI responses:

$$Repeatability\ NDVI = \frac{\sigma^2_{genotype}}{\sigma^2_{genotype} + \frac{\sigma^2_{error}}{n_{runs} \times n_{reps}}}$$

The following model was used to calculate repeatability for SPAD responses:

$$Repeatability\ SPAD = \frac{\sigma^2_{genotype}}{\sigma^2_{genotype} + \frac{\sigma^2_{genotype*run}}{n_{runs}} + \frac{\sigma^2_{error}}{n_{runs} \times n_{reps}}}$$

Plant Responses

A Beta distribution was assumed for NDVI based on the values being non-negative, continuous, and restricted to a finite interval of 0 to 1 that was skewed. The Beta distribution is flexible, relying on the variance and scale parameter resulting from a transformation, accommodates many distribution shapes, but specifically in this case, skewed right (Gbur et al., 2012; Gbur & Thompson, 2015). Much of the data were concentrated near a single point due to a moderate proportion of the genotypes having decreased response values (NDVI = ~0), and the surviving plants having a random distribution of values. The beta distribution can compensate for the lack of normality and the corresponding effect on the variance. To simplify the model, correlation of DAT intervals was assumed to be equal and therefore treated as the second split opposed to repeated measures. Given the scope of the objective, to rank genotypes based on overall response, a correlation of intervals would be unnecessarily complicate the model. An ANOVA for NDVI response was performed using all runs to determine how the various genotypes responded to the hypoxic treatment. Subsequently, a Tukey HSD was performed to rank the differences of the genotypes analyzed in the ANOVA. The same model as above was used to perform ANOVA for the SPAD data; however, a gamma distribution was assumed because the responses were measured using continuous, non-negative, and unrestricted values. Again, the same model was used to analyze data for the FDS responses. At the time of analysis, responses were pooled into three classes: 1-3 – tolerant (T), 4-6 – moderately tolerant (MT), and 7-9 – susceptible (S). Data were then analyzed based on an ordered multinomial distribution. The mean NDVI responses for each of the assigned classes were averaged and separated using a Tukey HSD test to validate the whether the use of a visual FDS is justified.

RESULTS

NDVI Response

The 33 diverse genotypes representing MG 4 to 7, potential sources of parental germplasm in the University of Arkansas breeding program, were evaluated for NDVI response following exposure to elevated CO_2 hypoxic conditions in a hydroponic system. Results of the ANOVA for NDVI response indicated no interaction effect for Genotype and DAT ($p=0.9948$). The NDVI response differed among genotypes ($p=0.0002$) and differed among DAT evaluation intervals ($p<0.0001$). It follows that genotypes could be consistently ranked based on NDVI independent of which DAT interval measurements were taken.

Due to the lack of interaction effect, genotypes were ranked using the Tukey HSD test following ANOVA (Table 1). Overall, responses did vary between DAT intervals, with intervals 9 and 0 DAT (0.298, 0.291), which did not differ from one another, producing larger mean NDVI values than 3 and 6 DAT (2.57, 2.45), which did not differ from one another (Table 2). Furthermore, run exhibited a covariance estimate of less than 0.4%, which explains a very small portion of the total variability observed, suggesting that the plant response was largely unaffected by time of year that screening was conducted. The repeatability of NDVI response was estimated at 94.5%. Interaction variance was zero and thus, excluded from the equation.

SPAD Response

The previously mentioned genotypes were evaluated for SPAD responses following the hypoxic treatment at the same time as NDVI responses. A significant difference was observed for Genotype ($p=0.0174$) and DAT ($p=<0.0001$) effects. However, results from the ANOVA indicated a significant interaction effect between Genotype and DAT ($p=<0.0001$) with crossover; therefore, the main effect could not be interpreted independent of DAT, as the SPAD

response differed among genotypes over time (Supplementary Table S3). This constraint of dependency disallows the use of SPAD responses to differentiate tolerance and susceptibility of germplasm with consistency. Furthermore, the SPAD response repeatability was found to be only 27.8%.

FDS Response

Using the three classes based on score groupings, FDS response was unaffected by genotype ($p=0.3369$), and no comparison and ranking were performed. However, consistent with the other evaluation methods of this study, FDS responses differed among DAT intervals ($p<0.0001$). Additionally, despite the ineffectiveness of this evaluation technique in a greenhouse, responses for all FDS ordered multinomial class (T, MT, S) least square means did correspond to significantly different mean NDVI values (Table 3).

DISCUSSION

A panel of 33 genotypes, replicated three times and repeated four times, were evaluated for foliar responses at four intervals following a hypoxic treatment using three phenotyping indices (NDVI, SPAD, and FDS). In each repetition of the experiment, genotypes consistently exhibited a spectrum of foliar responses ranging from total necrosis to asymptomatic as an effect of the hypoxic treatment. Average responses within an interval generally decreased following the termination of treatment; with chlorosis then necrosis symptoms worsening as time passed, for the first three intervals (0, 3, and 6 DAT) after cessation of CO_2 additions, and increased by the fourth interval (9 DAT) as tolerant varieties began to recover leaf area and leaves became greener. Of the three indices used to measure the effects of the hypoxic treatment, only NDVI was able to detect significant differences among genotypes independent of DAT. This was due to an interaction effect between genotype and DAT for the SPAD index and no significant genotype

effect for the FDS index. Besides being able to rank the responses of genotypes for selection, NDVI is fast (requiring mere seconds to gather data from a pot) , simple, and requires little labor inputs (no bending or handling of plants) or training, making the NDVI index an efficient option for rating plants.

Consistent with the objective of this study, results confirmed that germplasm could be screened for hypoxia tolerance, year-round, using hydroponic systems in a greenhouse, allowing for the culling of entries that exhibit greater susceptibility to CO_2/hypoxia damages. Because the seasonal timing of screening in a greenhouse had minimal effect on responses, screenings could be conducted year-round to increase the efficiency of identifying germplasm that is tolerant to low-oxygen environments. This reduction of time inputs would greatly accelerate the development of hypoxia-tolerant soybean cultivars in a soybean breeding program. Genetic variation is responsible for traits that determine the morphological and physiological mechanisms by which plants overcome hypoxic stress, such as root architecture or water and nitrogen use efficiency (Bailey-Serres &Voesenek, 2008; Valliyodan et al., 2017). Many of these adaptations allow plants to be robust to oxidative stresses imposed by hypoxia (Ahmed et al., 2013). This method was able to induce a range of foliar symptoms successfully and consistently in response to an elevated CO_2, O_2-deficient environment. These varied responses are explained by the diverse parental background of the genotypes and the presence or absence of traits to overcome hypoxia stress.

The absence of oxygen causes a series of reactions in plants: a reduction of stomatal conductance or restricted hydraulic conductivity in roots leads to reduced water uptake that is followed by an internal water deficit, which leads to reduced transpiration and photosynthesis (Else et al., 2001; Ashraf and Arfan, 2005; Araki, 2006; Mutava et al., 2015). These events are

ultimately manifested in the leaf as reduced chlorophyll content or tissue death (Ashraf et al., 2011). Therefore, foliar symptoms are a dependable indicator of hypoxia stress. An overall reduction of chlorophyll content and leaf canopy was observed following hypoxic treatment throughout this experiment, consistent with previous studies (Bacanamwo and Purcell, 1999a; Boru et al., 2003; Araki, 2006). Furthermore, exposure to hypoxic environments reduces nitrogen uptake and assimilation (Bacanamwo and Purcell, 1999b; Sullivan et al., 2001), further contributing to yellowing of leaves. For these reasons, SPAD and NDVI were selected as indices to observe changes in leaf greenness. The use of SPAD meters to document soybean greenness differences in response to hypoxia has been successful in previous studies resultant of decreased nitrogen uptake and chlorophyll production (Bacanamwo and Purcell, 1999; Boru et al., 2003; Araki, 2006; Board, 2008; Noulas et al., 2018), however, measurements were obtained at a single point in time, as temporal changes were beyond the scope of research objectives. Use of NDVI as a metric to determine differences between tolerant and susceptible varieties was successful due to varied levels of canopy greenness as a result of reduced nitrogen uptake and reduced photosynthesis, similar to conclusions of previous studies by Clark (2016), Hummer (2018), and Seo et al. (2019).

Hummer (2018) concluded that SPAD and NDVI were superior techniques to phenotype flood tolerance in field experiments due to greater broad-sense heritability, as well as being immune to the variance and error associated with objective human sampling. The current study was also able to conclude that the quantifiable sensor data were superior to data gathered using the FDS index. Beyond statistical advantages, NDVI also has some practical advantages over SPAD. The use of NDVI allows for the quick measurement of an entire pot from a height of ~0.5 m compared to taking measurements from each plant in a pot. Measuring each plant requires

more time, which becomes significant on the scale of a breeding trial; while SPAD evaluations lose precision due to environmental changes that occur during a longer evaluation period (Tattaris, Reynolds, & Chapman, 2015). The correlation of FDS classes to significantly different mean NDVI values validates the previous use of this index in field settings, but application of the index using the explored methodology was restricted, likely due to spatial scale. Furthermore, Bégué et al. (2010) reported was strongly correlated with SPAD values, supporting that it was an acceptable technique for evaluations considering the SPAD index's dependence of time of evaluation and reduced time efficiency; SPAD required handling of multiple plants and attention to consistent, uniform measurements taking up to a minute per pot compared to seconds for FDS and NDVI.

Of the 33 genotypes evaluated in this study, 14 were previously evaluated by Wu et al. (2017) in flooded-field conditions for multiple years. Six genotypes that were previously characterized as tolerant in flooded-field scenarios were also deemed to not differ from the most tolerant entry evaluated. Four genotypes previously designated as flood-sensitive did not differ from the most sensitive line in this study. Moreover, of the four genotypes with contrasting responses between the current research and the reports of Wu et al. (2017), only a single entry was selected as tolerant in the greenhouse setting and this genotype was characterized as susceptible in the field. Additionally, three genotypes previously characterized as susceptible by Wu et al. (2017) showed tolerance of hypoxic conditions in the greenhouse experiment. The latter observation requires further investigation. A proposed explanation for the contradictory responses is that field susceptibility could be linked to another variable present in the field environments, such as Oomycetes, including *Pythium* and *Phytophthora*. Both *Pythium* and *Phytophthora* are associated with cooler air and soil temperatures and rainfall that occur during

early vegetative stages of soybean (Rojas et al., 2017). Future selections will require screening for resistance/tolerance to pathogens frequently observed in flooded fields, such as *Pythium* and *Phytophthora* (Bowers et al.,1999; Rojas et al., 2017). There are overlapping symptoms for field-flood damages and Oomycete damages, such as plant stunting, root necrosis, and yellow leaves (Kirkpatrick, Rupe, & Rothrock, 2006). Thus, genotypes showing these symptoms in a flooded field may not be selected as they appear flood susceptible when they in fact may be tolerant to flood conditions, but susceptible to Oomycetes.

Greenhouses allow for intensive regulation of environmental factors that are difficult or even impossible to control in field experiments. Inconsistencies in germplasm responses within and across studies have been observed as outlined by Rhine et al. (2010), Carlin (2014), and Jitsuyama (2015). Conflicting results are attributed to highly variable environmental factors regardless of location. In the current study, the total variation explained by the covariate 'run' was less than 1% when using the NDVI index, and the repeatability of NDVI index was 94.5%, indicating the method used provides good reproducibility by controlling many environmental factors. This added level of control allows for the selection of traits for hypoxia tolerance, while excluding selection of tolerance of confounding factors in field evaluations.

Previous field studies have used evaluation protocols that use a subjective, visual score to rate soybean field-flooding tolerance and susceptibility (Wu et al., 2017; Hummer, 2018). Optical sensors, such as NDVI and SPAD meters, are objective, quantifiable, non-destructive, and highly efficient making them suitable for application in screening methods and data analysis (Edalat et al., 2019). Inefficiency of field observations are due to large labor inputs and slow generation time and are limited to a single assessment per planting per growth stage. The ability to separate genotypes based on evaluations in the greenhouse was dependent on timing of the

evaluation as well as the technique used (SPAD, NDVI, and FDS). For our purposes, NDVI was the most efficient technique to rank tolerance of germplasm compared to the other indices explored in this research. Moreover, there was no interaction between genotype and DAF ($p=0.9948$), removing dependence on evaluation time interval. This lack of temporal dependence allows for the acceleration of screenings by shortening each cycle time by six to nine days.

There is an immediate opportunity to apply these results to benefit a breeding program. Prior investigations have focused primarily on effects of flooding during late vegetative and early reproductive growth stages (Rhine et al., 2010; Kuswantoro, 2011), as R1 and after is when soybean plants are most sensitive to waterlogging (Linkemer, 1998). It is possible to identify genes associated with plant tolerance to hypoxic environments. This can be accomplished by creating crosses using varieties identified in this study as tolerant or susceptible. The resulting offspring of those crosses can be advanced to the F_4 breeding population, at which point single plant selections can be performed for use in F_5 progeny rows with the purpose of creating a genetic map to identify quantitative trait loci and single nucleotide polymorphisms associated with hypoxia tolerance.

The greatest proportion of extreme rainfall events occur during the planting and early vegetative portion of the growing season. Currently, MG 4 lines are being developed to be grown throughout the Midsouthern U.S., including Arkansas because MG 4 varieties allow comparable yields to MG 5 varieties with reduced inputs when planted at an optimal planting date in April (Salmerón et al., 2014; Salmerón et al., 2016). To maximize yields of MG 4 lines, the optimal planting date is in April, prior to the peak monthly average rainfall of May (National Weather Service – NOAA, 2020). Incorporation of hypoxia-tolerant traits identified in a greenhouse setting could better protect plants that are planted prior to the spring rains in Arkansas. Due to

the heavy soil textures, with a greater proportion of clay that cause the region to be prone to flood throughout the growing season (Scott et al., 1989), fields are also slow to dry following rains in the cooler autumn seasons. Currently, MG 4 lines that are not adapted are at risk of substantial to total stand losses. Losses that could be mitigated by adapting high-yielding varieties to environmental stressors, such as flood. The findings of this research can be applied to a breeding program to readily identify genotypes with tolerance to low-O_2 environments that develops under prolonged flooding in fields to develop improved cultivars.

Vegetative evaluations require only five weeks to complete a cycle, therefore, the inclusion of off-season screenings allow for an increase in total replicated evaluations compared to field studies. Reproductive evaluations using this method of greenhouse screening would require supports and additional management to prevent lodging, as plants would be large and unwieldy. Thus, field screening will be required to assess greenhouse selections for yield potential. As previously mentioned, selections for pathogen tolerance will be required following greenhouse selection for tolerance to hypoxic environments. While this study demonstrated the potential to increase efficiency through short cycle times and year-round observations, the method still relied on human labor to conduct evaluations.

There is an opportunity to further develop the methodology investigated in this research by increasing time-efficiency and optimizing the rate and duration of the hypoxia treatment. A first step to elevate time-efficiency would be to implement automated remote sensing. Carrier hardware and imaging subsystems utilizing radio-frequency identification (RFID) have previously been reported, in which modified sprayer booms are altered to carry multi-spectral sensors and may be controlled manually or automatically (Yang et al., 2014). The use of an automated system would allow for optimal timing and consistency of observations, as additional

sensors provide information about air temperature, humidity, and lighting, the last of which is important for any spectral measurements, as light may cause unwanted interference (Ehret, Lau, Bittman, Lin, & Shelford, 2001). Implementation of automated remote sensing would reduce labor inputs and eliminate human errors, while improving precision during data gathering opposed to proximal sensing (Csornai et al., 1999; Tattaris et al., 2015). Secondly, optimization of the hypoxia treatment by establishing critical values for rate and duration to induce a response in soybean could better improve selections using the methods described herein. Currently, there have been no studies to optimize the rate and duration of a hypoxic treatment to induce signaling and response in *Glycine max*. Boru et. al (2003) used various CO_2 rates under a single duration (14 d); however, the purpose was to observe morphological and physiological changes, not establish critical rates or durations. Carrying out such an experiment using response-surface design could theoretically identify optimal rates and durations of the hypoxia treatment.

Use of the system prior to a spring planting could reduce costs by minimizing the total number of entries in a yield trial if susceptible selections were excluded following off-season prescreening. Genotypes selected for hypoxia tolerance at early vegetative growth stages may prove to be robust to yield losses, where Reyna et al. (2003) noted a negative correlation between waterlogging injury and yields following waterlogging of genotypes evaluated in reproductive stages. Further evaluations are required to determine a correlation between observed vegetative injury and final yields following waterlogging stress using germplasm selected for hypoxia tolerance in a greenhouse.

Finally, there are other vegetative indices not explored in this research that should be examined in a future study. Red-edge NDVI (reNDVI) has been shown to have more robust performance over SPAD or NDVI, as reDNVI is insensitive to vegetative coverage and

environmental areas not covered by vegetation (Deng et al., 2018). Dark green color index (DGCI) is another alternative index that may prove to be advantageous over NDVI because DGCI corresponds well to the SPAD index and can be used remotely (Rorie et al., 2011).

CONCLUSIONS

As a flood-sensitive crop, soybean has been observed to have adaptations to hypoxic, waterlogged environments. Identification of germplasm with these traits is essential to develop high-yielding cultivars that can withstand periodic, seasonal flooding in an agronomic production system. The application of a greenhouse germplasm screening protocol helped to effectively differentiate plants that were tolerant and susceptible to low-oxygen, elevated-CO_2 conditions in early vegetative growth stages in a hydroponic system. The use of such a protocol was effective throughout the year due to being robust to seasonal variation of air temperature, light intensity, and climate. The five-week duration of the experiment allowed for multiple cycles of screening throughout the year opposed to a singular evaluation under field conditions.

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TABLES AND FIGURES

Table 1. Separation of genotypes based on mean normalized difference vegetation index responses (NDVI).

Genotype	LS Mean NDVI	Std. Err.	Lower 95% CL	Upper 95% CL										
R11-6870	0.363	0.052	0.268	0.471	A									
Walters	0.342	0.050	0.251	0.446	A	B								
RA-452	0.339	0.050	0.249	0.444	A	B	C							
PI 471938	0.331	0.050	0.241	0.436	A	B	C	D						
R04-342	0.318	0.048	0.231	0.420	A	B	C	D	E					
AG56X8	0.312	0.048	0.226	0.412	A	B	C	D	E	F				
Ozark	0.309	0.048	0.224	0.409	A	B	C	D	E	F	G			
R11-2915	0.307	0.051	0.217	0.415	A	B	C	D	E	F	G	H		
PI 341257	0.301	0.047	0.218	0.401	A	B	C	D	E	F	G	H		
R99-1613F	0.300	0.050	0.212	0.406	A	B	C	D	E	F	G	H		
R06-4433	0.299	0.047	0.216	0.399	A	B	C	D	E	F	G	H		
UA 4805	0.292	0.047	0.209	0.393	A	B	C	D	E	F	G	H		
PI 564261	0.291	0.046	0.209	0.390	A	B	C	D	E	F	G	H		
S99-2281	0.289	0.048	0.205	0.391	A	B	C	D	E	F	G	H		
UA 5612	0.288	0.046	0.206	0.385	A	B	C	D	E	F	G	H		
R07-6669	0.284	0.045	0.203	0.381	A	B	C	D	E	F	G	H		
91210-350	0.283	0.045	0.203	0.380	A	B	C	D	E	F	G	H		
R15-10832	0.277	0.045	0.198	0.373	A	B	C	D	E	F	G	H	I	
R09-4095	0.274	0.054	0.182	0.391	A	B	C	D	E	F	G	H	I	J
PI 574476A	0.268	0.044	0.191	0.363		B	C	D	E	F	G	H	I	J
R10-230	0.263	0.043	0.187	0.356			C	D	E	F	G	H	I	J
R10-2379	0.256	0.044	0.179	0.353				D	E	F	G	H	I	J
UA 5014C	0.252	0.043	0.177	0.346				D	E	F	G	H	I	J
AG55X7	0.248	0.042	0.175	0.339					E	F	G	H	I	J
R10-4892	0.238	0.042	0.166	0.329						F	G	H	I	J
R02-6268F	0.233	0.041	0.162	0.323							G	H	I	J
PI 408105A	0.227	0.043	0.153	0.323							G	H	I	J
R11-3283	0.222	0.040	0.152	0.311								H	I	J
PI 221BB	0.221	0.043	0.148	0.316								H	I	J
S11-25108	0.220	0.043	0.146	0.317								H	I	J
N94-7440	0.210	0.037	0.146	0.293									I	J
S12-1362	0.203	0.040	0.135	0.294									I	J
S11-25615	0.199	0.037	0.137	0.282										J

Genotypes connected by the same letter are not significantly different at p=0.05 as determined by Tukey honest significant difference (HSD) test.

Table 2. Days after treatment (DAT) mean NDVI responses.

DAT	LS Mean NDVI	Std. Err.	Lower 95% CL	Upper 95% CL		
9	0.298	0.038	0.229	0.378	A	
0	0.291	0.038	0.223	0.371	A	
3	0.257	0.035	0.195	0.332		B
6	0.245	0.034	0.185	0.317		B

DAT intervals not connected by the same letter are significantly different at p=0.05

Table 3. Foliar damage score (FDS) class vs. NDVI Tukey HSD ranking.

Level	Mean NDVI			
Tolerant (FDS= 1-3)	0.388	A		
Moderate (FDS= 3-6)	0.328		B	
Susceptible (FDS= 6-9)	0.207			C

Levels not connected by the same letter are significantly different at p=0.05.

(A) R11-6870 Run 1, Bench 3, 3 DAT

(B) R11-3283 Run 1, Bench1, 3 DAT

Figure 1. (A) Image shows tolerant variety (R11-6870) response to CO_2 treatment 3 days after termination of treatment and **(B)** Image shows susceptible variety (R11-3283) response to CO_2 treatment 3 days after termination of treatment.
Photos by author.

APPENDIX

Table S1. Stock fertilizer solution formula.

STOCK	CHEMICAL CONSTITUENTS	STOCK PREPARATION	REQUIRED CONCENTRATE	SOLUTION/LITER OF SYSTEM WATER
A	$Ca(NO_3)_2 \cdot 4H_2O$	826.4 g/L	3.5 M	1 mL/1 L
B	KNO_3	252.8 g/L	2.5 M	1 mL/1 L
C	KH_2PO_4	136.1 g/L	1 M	1 mL/1 L
D	$MgSO_4 \cdot 7H_2O$	246.5 g/L	1 M	1 mL/1 L
E	H_3BO_3	2.8 g/L	45.2 mM	1 mL/1 L
	$MnSO_4 \cdot H_2O$	1.8 g/L	9.09 mM	1 mL/1 L
	$ZnSO_4 \cdot 7H_2O$	.2 g/L	695.5 mM	1 mL/1 L
	$NH_4MoO_4 \cdot 4H_2O$	.022 g/L	171.1 mM	1 mL/1 L
	$CuSO_4 \cdot 5H_2O$	.1 g/L	400 mM	1 mL/1 L
F	Fe DTPA 11%	11.25 g/L		1 mL/1 L

Table S2. Summary table of genotypes evaluated and previous tolerance/susceptibility designation from field screening.

Entry	Name	Previous Rating	Entry	Name	Previous Rating
1	R10-230	Tolerant	18	S11-25108	Tolerant
2	R10-4892	Tolerant	19	S12-1362	Tolerant
3	R13-12552	Tolerant	20	S11-25615	Sensitive
4	R07-6669	Tolerant	21	S99-2281	Sensitive
5	R11-6870	Tolerant	22	91210-350	Tolerant
6	Walters	Tolerant	23	PI 408105A	Tolerant
7	R04-342	Tolerant	24	PI 221BB	Tolerant
8	UA 5014C	Sensitive	25	PI 471938	Tolerant
9	R06-4433	Sensitive	26	PI 574476A	Tolerant
10	R99-1613F	Sensitive	27	PI 341257	Tolerant
11	Ozark	Sensitive	28	PI 564261	Tolerant
12	UA 4805	Sensitive	29	RA-452	Tolerant
13	R11-3283	Sensitive	30	N94-7440	Tolerant
14	R10-2379	Sensitive	31	UA 5612	Tolerant
15	R11-2915	Sensitive	32	R15-10832	Tolerant
16	R09-4095	Sensitive	33	AG55X7	
17	R02-6268F	Sensitive	34	AG56X8	

Table S3. Ranking of genotypes*days after treatment based on least square means.

Conservative T Grouping for Genotype*DaysAfter Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Genotype	DaysAfter	Estimate										
R06-4433	0	4.4413	A									
UA 5612	0	4.2064	A	B								
R04-342	0	4.1832	A	B	C							
S99-2281	0	4.1367	A	B	C	D						
R11-6870	0	4.1234	A	B	C	D						
PI 471938	0	4.0281	A	B	C	D						
RA-452	0	3.9815	A	B	C	D						
AG56X8	0	3.971	A	B	C	D						
S11-25108	0	3.9129	A	B	C	D	E					
91210-350	0	3.7628	A	B	C	D	E					
R99-1613F	0	3.5777	A	B	C	D	E					
R10-2379	0	3.419	A	B	C	D	E	F				
UA 4805	0	3.3851	A	B	C	D	E	F				
PI 574476A	0	3.3227	A	B	C	D	E	F				
R15-10832	0	3.2639	A	B	C	D	E	F	G			
R10-230	0	3.2351	A	B	C	D	E	F	G			
R11-2915	0	3.116	A	B	C	D	E	F	G	H		
Walters	0	3.1102	A	B	C	D	E	F	G	H		
R10-2379	9	3.0042	A	B	C	D	E	F	G	H		
N94-7440	0	2.9975	A	B	C	D	E	F	G	H		
Ozark	0	2.9912	A	B	C	D	E	F	G	H		
UA 5014C	0	2.808	A	B	C	D	E	F	G	H	I	
PI 574476A	3	2.7573	A	B	C	D	E	F	G	H	I	
AG55X7	0	2.7455	A	B	C	D	E	F	G	H	I	
PI 341257	0	2.6842	A	B	C	D	E	F	G	H	I	J
R10-2379	6	2.656	A	B	C	D	E	F	G	H	I	J
PI 564261	0	2.6461	A	B	C	D	E	F	G	H	I	J
R10-2379	3	2.6415	A	B	C	D	E	F	G	H	I	J
S12-1362	0	2.609	A	B	C	D	E	F	G	H	I	J	K
R11-6870	3	2.6068		B	C	D	E	F	G	H	I	J	K
S99-2281	3	2.5685		B	C	D	E	F	G	H	I	J	K
PI 564261	9	2.5032		B	C	D	E	F	G	H	I	J	K
PI 408105A	0	2.4886		B	C	D	E	F	G	H	I	J	K
PI 471938	9	2.488			C	D	E	F	G	H	I	J	K
S11-25615	0	2.4864			C	D	E	F	G	H	I	J	K
R07-6669	0	2.4813				D	E	F	G	H	I	J	K

Table S3. (Cont.)

Conservative T Grouping for Genotype*DaysAfter Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

Genotype	DaysAfter	Estimate											
PI 574476A	9	2.448				D	E	F	G	H	I	J	K
AG56X8	9	2.3947				D	E	F	G	H	I	J	K
R11-3283	9	2.3788				D	E	F	G	H	I	J	K
AG56X8	3	2.3316					E	F	G	H	I	J	K
R11-6870	9	2.3205					E	F	G	H	I	J	K
R04-342	3	2.2828					E	F	G	H	I	J	K
R11-6870	6	2.2771					E	F	G	H	I	J	K
PI 574476A	6	2.2478					E	F	G	H	I	J	K
R11-3283	0	2.1856	L				E	F	G	H	I	J	K
R04-342	6	2.1645	L				E	F	G	H	I	J	K
PI 341257	9	2.1639	L				E	F	G	H	I	J	K
PI 471938	6	2.1602	L				E	F	G	H	I	J	K
PI 341257	6	2.1494	L				E	F	G	H	I	J	K
R11-3283	3	2.14	L				E	F	G	H	I	J	K
Ozark	3	2.1389	L				E	F	G	H	I	J	K
PI 341257	3	2.1168	L				E	F	G	H	I	J	K
R10-230	6	2.0736	L				E	F	G	H	I	J	K
PI 471938	3	2.07	L	M			E	F	G	H	I	J	K
PI 221BB	0	2.0639	L	M			E	F	G	H	I	J	K
UA 5612	3	2.0548	L	M			E	F	G	H	I	J	K
R04-342	9	1.9793	L	M			E	F	G	H	I	J	K
RA-452	9	1.9634	L	M			E	F	G	H	I	J	K
UA 5612	6	1.9396	L	M			E	F	G	H	I	J	K
RA-452	6	1.9239	L	M			E	F	G	H	I	J	K
R10-230	9	1.9164	L	M			E	F	G	H	I	J	K
R06-4433	3	1.9072	L	M			E	F	G	H	I	J	K
R10-4892	0	1.9052	L	M			E	F	G	H	I	J	K
Walters	6	1.9033	L	M			E	F	G	H	I	J	K
R10-230	3	1.8709	L	M			E	F	G	H	I	J	K
Walters	9	1.8605	L	M			E	F	G	H	I	J	K
UA 5612	9	1.8263	L	M	N		E	F	G	H	I	J	K
UA 5014C	3	1.8149	L	M	N		E	F	G	H	I	J	K
S12-1362	3	1.7822	L	M	N	O	E	F	G	H	I	J	K
RA-452	3	1.7762	L	M	N	O		F	G	H	I	J	K
Ozark	9	1.7758	L	M	N	O		F	G	H	I	J	K
Ozark	6	1.7563	L	M	N	O		F	G	H	I	J	K
Walters	3	1.7126	L	M	N	O		F	G	H	I	J	K
PI 564261	6	1.6959	L	M	N	O		F	G	H	I	J	K
R02-6268F	0	1.6385	L	M	N	O		F	G	H	I	J	K

Table S3. (Cont.)

Conservative T Grouping for Genotype*DaysAfter Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

PI 564261	3	1.6037	L	M	N	O		F	G	H	I	J	K	
PI 408105A	3	1.5926	L	M	N	O		F	G	H	I	J	K	
R07-6669	6	1.5784	L	M	N	O			G	H	I	J	K	
R06-4433	9	1.564	L	M	N	O			G	H	I	J	K	
R07-6669	9	1.5513	L	M	N	O			G	H	I	J	K	
PI 408105A	9	1.4554	L	M	N	O	P		G	H	I	J	K	
R07-6669	3	1.4476	L	M	N	O	P			H	I	J	K	
UA 5014C	9	1.4342	L	M	N	O	P			H	I	J	K	
S99-2281	9	1.4267	L	M	N	O	P			H	I	J	K	
S99-2281	6	1.4257	L	M	N	O	P			H	I	J	K	
UA 5014C	6	1.4157	L	M	N	O	P			H	I	J	K	
91210-350	3	1.4118	L	M	N	O	P			H	I	J	K	
UA 4805	3	1.4082	L	M	N	O	P			H	I	J	K	
PI 408105A	6	1.3908	L	M	N	O	P	Q		H	I	J	K	
R99-1613F	3	1.3499	L	M	N	O	P	Q		H	I	J	K	
UA 4805	6	1.3123	L	M	N	O	P	Q		H	I	J	K	
AG55X7	9	1.2494	L	M	N	O	P	Q		H	I	J	K	
91210-350	9	1.0679	L	M	N	O	P	Q	R	H	I	J	K	
UA 4805	9	1.056	L	M	N	O	P	Q	R	H	I	J	K	
R99-1613F	6	1.0447	L	M	N	O	P	Q	R	H	I	J	K	
R09-4095	6	1.0179	L	M	N	O	P	Q	R	H	I	J	K	S
PI 221BB	9	1.0104	L	M	N	O	P	Q	R		I	J	K	S
R10-4892	6	0.984	L	M	N	O	P	Q	R		I	J	K	S
R10-4892	3	0.9485	L	M	N	O	P	Q	R		I	J	K	S
R02-6268F	3	0.9381	L	M	N	O	P	Q	R		I	J	K	S
91210-350	6	0.9348	L	M	N	O	P	Q	R		I	J	K	S
AG55X7	3	0.9188	L	M	N	O	P	Q	R		I	J	K	S
R09-4095	9	0.8804	L	M	N	O	P	Q	R		I	J	K	S
S11-25108	3	0.8788	L	M	N	O	P	Q	R		I	J	K	S
S12-1362	9	0.858	L	M	N	O	P	Q	R		I	J	K	S
R15-10832	6	0.8282	L	M	N	O	P	Q	R		I	J	K	S
R15-10832	9	0.8231	L	M	N	O	P	Q	R		I	J	K	S
R09-4095	0	0.8076	L	M	N	O	P	Q	R		I	J	K	S
S11-25108	6	0.805	L	M	N	O	P	Q	R			J	K	S
S12-1362	6	0.8005	L	M	N	O	P	Q	R			J	K	S
R11-2915	9	0.7621	L	M	N	O	P	Q	R			J	K	S
R15-10832	3	0.7514	L	M	N	O	P	Q	R			J	K	S
R02-6268F	9	0.7454	L	M	N	O	P	Q	R			J	K	S
R99-1613F	9	0.7219	L	M	N	O	P	Q	R			J	K	S
R09-4095	3	0.6692	L	M	N	O	P	Q	R			J	K	S

Table S3. (Cont.)

Conservative T Grouping for Genotype*DaysAfter Least Squares Means (Alpha=0.05)

LS-means with the same letter are not significantly different.

R11-2915	6	0.6512	L	M	N	O	P	Q	R	K	S
R02-6268F	6	0.6384	L	M	N	O	P	Q	R	K	S
S11-25108	9	0.4985	L	M	N	O	P	Q	R	K	S
R11-2915	3	0.489	L	M	N	O	P	Q	R		S
R10-4892	9	0.4059		M	N	O	P	Q	R		S
N94-7440	3	0.1808			N	O	P	Q	R		S
PI 221BB	6	0.01913			N	O	P	Q	R		S
PI 221BB	3	-0.1573				O	P	Q	R		S
N94-7440	6	-0.3138					P	Q	R		S
S11-25615	3	-0.3975					P	Q	R		S
S11-25615	6	-0.4654						Q	R		S
S11-25615	9	-0.6122							R		S
N94-7440	9	-0.8014									S

OVERALL CONCLUSION

Plant traits that allow for tolerance to CO_2-rich, low-O_2 environments are complex and influenced by many factors associated with the plant itself as well as the environment. Plant characteristics that influence their ability to withstand hypoxic environments include the growth stage at which the stress occurs, plant architecture, specifically roots, and the plant's ability to undergo metabolic changes that allow for anaerobic respiration. The environmental factors that influence plant responses include the soil texture in which the plant is growing when the stress occurs, air temperature, the duration and severity of the hypoxic stress (i.e. flooding), and the presence of root-rot pathogens. The results of this study showed that the herein outlined methods of germplasm screening can differentiate plant responses based on foliar changes following hypoxic stress. Furthermore, our research indicated that it is possible to create hypoxic stress by addition of CO_2 gas to a hydroponic environment that produces chlorotic leaves, consistent with plant responses observed under flooded field conditions. Hydroponic systems offer the advantage of a soilless media, but with standard substrate used in such systems require transplantation. This study demonstrated that clay pebbles is a suitable alternative media to rockwool pellets, the industry standard, and has the added advantage of direct sowing.

Leaf chlorotic response is an indicator of hypoxic stress in plants and may be measured through the use of multiple indices, such as foliar damage score (FDS), normalized difference vegetation index (NDVI), and soil-plant analysis development (SPAD); all of which are based on plant greenness levels that are directly associated with plant chlorophyll content. The results of this study showed that the responses of plants to the CO_2 induced hypoxic treatment may be substantial enough to be measured using these indices. However, there is a marked advantage to the use of NDVI as the primary phenotyping index due to its simplicity and accuracy regarding

measuring the contrasting differences of diverse germplasm in response to hypoxic conditions. The use of the method holds potential as a tool to aid the production of flood-tolerant cultivars in a breeding program, as germplasm that is tolerant to low-O_2, elevated-CO_2 conditions may offer similar tolerances to flooded conditions.