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Increasing The Agronomic And Economic Value Of Chickpea And Pea

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INCREASING THE AGRONOMIC AND ECONOMIC VALUE OF CHICKPEA AND PEA

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ABSTRACT

Domestication has had a profound global impact on human history and a wide range of plants. Understanding the advertent and inadvertent effects of domestication on crops has been instrumental in bolstering food security efforts. For instance, by identifying and re-incorporating lost genotypic variation due to domestication, we can increase crop tolerance to biotic and abiotic stressors. With changing climatic conditions and the ever-growing human population, it has become more imperative to increase and fortify agricultural production. My dissertation addresses this topic in two agronomically important legumes: chickpea (*Cicer arietinum*) and pea (*Pisum sativum*). My research aims to increase the agronomic and economic value of these legumes to facilitate agricultural production as well as lessen financial burdens to farmers. To accomplish this aim, in chickpea, we identified the physiological and genetic basis of the green-seed market type and identified the effects of domestication on the response to a novel environment. Furthermore, in pea, we investigated phenotypic variation for cover cropping traits using wild accessions, landraces, and modern varieties.

In chickpea, we identified that green-seeded chickpea market type was due to a loss of function mutation of the CaStGR1 (carietinum stay-green gene 1) gene involved in chlorophyll catabolism. Additionally, physiological testing in drought conditions revealed this phenotype to be of the "cosmetic" and not the "functional " stay-green variety. Furthermore, nutritional analysis revealed that this trait was associated with a 2-3 fold increase in provitaminogenic carotenoids that are important for human nutrition. Therefore, this green-seeded trait may increase both the economic and nutritional value of chickpea.

To identify how domestication has affected chickpea response to novel environments, we took a whole-plant approach and measured above- and below-ground response to increased nitrogen presence in chickpea. Results revealed that domestication has canalized domesticated chickpea response to nitrogen-rich environments. Furthermore, the variable response of wild chickpea to nitrogen illustrated the need for the use of a comprehensive assortment of wild relative accessions to fully discern the effects of domestication on domesticated organisms.

Lastly, we coined the terms "rotational" and "intercropping value" and provide a mathematical equation to quantify these terms. We also discuss numerous methods on how to increase these values. To demonstrate these ideas, we measured the rotational values of domesticated and wild pea. We identified that rotational values and cover-cropping traits such as nutrient mobilization and microbial recruitment vary within field pea. These results indicate that field pea could potentially be improved as a rotational partner and that the use of wild relatives in cover cropping research, which has been underutilized, should be considered.

Overall, these results illustrate the importance of understanding the effects of domestication and highlights the importance of crop wild relatives as phenotypic reservoirs for crop improvement. Collectively, my research provides insightful information that can facilitate agricultural production at the farming and breeding level.

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CHAPTER 1: INTRODUCTION

1.1 Introduction

The practice of domestication was a pivotal innovation in human history that allowed for the rise of modern civilization (Diamond, 2002). Domestication— or the rapid directional modification of plants and animals to benefit humans—laid the foundation for agriculture, which enabled humans to shift from a nomadic to a sedentary lifestyle (Diamond, 2002). Since the beginning of domestication during the Neolithic period 12,000 years ago, humans have domesticated hundreds of animal and plant species (Meyer et al., 2012). Currently, three domesticated species (rice, corn, and wheat) provide 60% of the world's food energy intake (FAO, 2018).

In addition to being a foundational cornerstone of our society, domestication has played an integral role in our understanding of ecology and evolutionary biology. For instance, in the *Origin of Species* (1859), Darwin illustrated that humans have strongly modified the phenotypic traits of domesticated species, such as rock pigeons, ancon sheep, cabbages, and gooseberries, and concluded that nature could have similar phenotypic effects on organisms over a much longer timescale. Darwin's acute observations and experimentation of domesticated species directly impacted our understanding of evolution and was a fundamental component in the modern synthesis (Huxley, 1942)—a mathematical framework that combined Darwin's theory of natural selection and Mendelian inheritance to explain how organisms evolve. More recently, the study of domesticated organisms has helped address a suite of questions around epigenetics (reviewed in Shi and Lai, 2015; Ding and Chen, 2018), gene flow (Coulibaly et al., 2002;

Ellstrand et al., 1999), genome evolution (Miller et al., 2000; Verde et al., 2013), extended evolutionary synthesis (Piperno et al. 2017), local adaptation (Ross-Ibarra et al. 2007; Olsen and Wendel, 2013), and food security (Mayes et al., 2012; Warschefsky et al. 2014).

This chapter explores the inadvertent and advertent effects of domestication on plants, specifically discussing the effect of domestication on genetic and phenotypic diversity in crops, and synthesizing recent studies characterizing domestication's effect on plant belowground traits such as root morphology and plant-microbe interactions. This is followed by a discussion of the current obstacles that hinder agricultural production and the potential solutions offered by belowground traits. We then conclude with how crop wild relatives may be useful tools for reducing the inadvertent effects of domestication to potentially increase agricultural production. Lastly, an outline of the dissertation is presented, and its intellectual merit is addressed through a comprehensive review of the current gaps in the literature on domestication and agronomy that this research aims to fill.

1.2 The Effect of Domestication on Plants

1.2.1 Genetic Diversity

Domestication has had advertent and inadvertent effects on organisms, with the result that some organisms have been so significantly altered that they are unrecognizable when compared to their wild progenitors (Doebely et al., 2006). In general, two overarching trends have emerged in domestication studies: 1) domestication typically reduces genetic diversity and 2) while increasing phenotypic diversity (Doebely et al., 2006; Gepts, 2004; Meyer and Purugganan, 2013). This is because domestication acts as a genetic bottleneck that reduces allelic variation within the gene pool (Doebely et al.,

2006; Gepts, 2004; Wright et al., 2005; Yamasaki et al., 2007). During the domestication process, the effective population size (Ne) of organisms is significantly reduced; where only a few individuals from a larger population are selected and allowed to reproduce because they have a desirable phenotype(s) (Wright et al., 2005; Meyer and Purugganan, 2013). For instance, the effective population size of cultivated chickpea, *Cicer arietinum*, (Ne = 2 K) is 100 times less than its progenitor *Cicer reticulatum* (Ne = 20 K) (von Wettberg et al., 2018). Thus, this reduction in effective population size coincides with a reduction in genetic diversity (Doebely et al., 2006; Meyer and Purugganan, 2013; Wright et al., 2005; Yamasaki et al., 2007).

Furthermore, the exertion of strong positive selection on favorable phenotypes leads to a reduction in nucleotide diversity near alleles/genes controlling these phenotypes; a process commonly referred to as a selective sweep (Shi and Lai, 2015; Tang et al., 2010; Yamasaki et al., 2007). Selective sweeps are commonly found in the genomes of domesticated organisms. For example, in maize, large blocks of reduced nucleotide diversity have been observed around agronomically important genes that control for endosperm color (Palaisa et al., 2004), branching (Clark et al., 2004; Camus-Kulandaivelu et al., 2008), and dwarfism (Camus-Kulandaivelu et al. 2008). Additionally, in rice, selective sweeps were largely responsible for the differentiation of *japonica* and *indica* cultivars (Olsen et al. 2006; Yang et al., 2017). Together, selective sweeps and genetic bottlenecks significantly reduce genetic diversity in modern-day crops (Shi and Lai, 2015), leading to negative inadvertent effects on tolerance traits against pests (Fontes-Puebla and Bernal, 2019), disease (Arora et al.,2019), and climate change (Raza et al., 2019)(explained in more detail below).

1.2.2 Phenotypic Diversity

Conversely, phenotypic diversity has increased due to domestication, with domesticated organisms displaying a greater range of phenotypic variation when compared to their wild progenitors (Darwin, 1867; Doebley et al., 2006). Selection for exaggerated phenotypes, such as enlarged fruit or seed size, and maladaptive wild phenotypes, like seed indehiscence and reduced seed dormancy (Meyer and Purugganan, 2013), greatly alters domesticated organisms, making them morphologically different from their wild progenitors (Doebley et al., 2006). For instance, the wild relative of maize was highly debated and went undiscovered for many years due to the large contrasting morphological differences between the two species, including female inflorescences (ears) size, reduced branching, the presence of casings surrounding the kernels, and seed indehiscence (Wilkes, 1967; Doebley et al., 1990). It was only after genomic advances that the debate was settled; molecular evidence from isozymes and chloroplast DNA revealed that the ancestral taxon of maize was teosinte, *Zea mays ssp. parviglumis*, and phylogenies revealed that maize arose from a single domestication event in the highlands of Mexico (Doebley, 1990; Matsuoka et al., 2002).

Another trend that has been identified in domestication studies is that domesticated organisms typically share a suite of common phenotypic characteristics known as the domestication syndrome (Hammer et al. 1984). In plants, these traits usually comprise a reduction in seed dormancy, plant height, toxins, seed shattering/fruit abscission, increase in seeds/fruit size, and a loss of vernalization (Hammer et al. 1984; Doebley et al. 2006; Lenser and Theißen, 2013). This suite of characteristics is believed to be caused by human-

mediated selection for traits that allow for efficient cultivation, such as reduced plant height and seed shattering, as well as culinary or aesthetic preferences such as color and taste (Lenser and Theißen, 2013). This common set of phenotypes found across many domesticated species are believed to be a result of convergent molecular mechanisms, such as mutations at orthologous loci (homologous genes that generally maintain a similar function to that of the ancestral gene) (Lenser and Theißen, 2013; Lin et al., 2012; Paterson et al., 1995). For instance, seed indehiscence in cereals, sorghum, rice, and maize, are all controlled by orthologous Sh1 genes (Paterson et al., 1995; Lin et al. 2012). Although there is evidence to support molecular convergence, this topic is still highly debated, with some evidence suggesting that non-orthologous loci can also be the basis of convergent phenotypes in domesticated species (Sood et al., 2002; Li and Gill, 2006; Rau et al., 2019; Sang, 2009).

1.2.3 Belowground Traits

The majority of plant domestication research has focused on its effects on aboveground traits. This bias has led to little insight into the effect of domestication on belowground traits such as root architecture and plant-microbe interactions. Only recently have belowground traits become a focus, due to advances in technological and methodological approaches that allow for accurate characterization of plant microbiome diversity and root architecture (reviewed in Tracy et al., 2020). Thanks to these advances, our knowledge about belowground traits has greatly increased over the past 30 years.

One of the first studies to address how domestication affects belowground traits was conducted by Kapulnik and Kushnir (1991), who focused on plant-mycorrhizal

interactions. They grew wild, primitive (landraces), and modern wheat lines inoculated with *Glomus intraradice*s, a vesicular-arbuscular mycorrhizal fungus (AMF), and recorded phenotypic differences. Kapulnik and Kushnir (1991) discovered that mycorrhizae dependence, the amount of plant growth that can be attributed to mycorrhizal symbiosis, was lower in modern wheat varieties than in wild or primitive varieties. The weakened plant-mycorrhizal relationship observed in wheat has also been recorded in breadfruit (Xing et al., 2012). Xing et al. (2012) noted that wild breadfruit is colonized more regularly by AMF than its modern relatives. Thus, it seems that domestication has significantly altered and weakened plant-mycorrhizal relationships in all crops. However, Lehmann et al. (2012) demonstrated in a meta-analysis that modern cultivars are more mycorrhiza-responsive than ancestral genotypes. But, this conclusion should not be weighed too heavily, since a direct comparison of modern cultivars to their wild progenitors was not made in most of the experiments included in the meta-analysis. In summary, more research is required to fully elucidate the effects of domestication on plant-mycorrhizal interactions.

Plant-mycorrhizae relationship is not the only symbiotic partnership that has potentially been affected by domestication. Plant-rhizobial interactions may also have been impacted. Mutch and Young (2004) noted that wild legumes (*Vicia and Lathyrus*) had higher nodule rhizobia diversity than cultivated pea (*Pisum sativa*) and broad bean (*Vicia faba*) when grown in the same environment. This study indicates that domestication has limited the rhizobia that can interact with domesticated legumes. However, a direct comparison between cultivated and wild progenitors, a more powerful method for addressing this question, was not made. A study that did take this approach was Kim et al. (2014), which involved growing wild (*Cicer reticulatum*) and cultivated chickpea (*Cicer*

arietinum) in an agricultural setting and measuring nodule rhizobia diversity. Kim et al. (2014) concluded that nodule rhizobia diversity was higher in wild chickpea than in cultivated chickpea. However, the limited data set of this study has called into question its applicability. In a more recent study, Greenlon et al. (2019) found that the most important factors influencing rhizobia diversity in chickpea nodules are edaphic and environmental factors, such as soil type and latitude. Therefore, it appears that domestication and breeding may have restricted the rhizobia that can interact with legumes, with environmental factors and latitude having a more influential effect on plant-rhizobial interactions.

Furthermore, recent research has demonstrated that domestication has also impacted plant rhizosphere communities. Szoboszlay et al. (2015) addressed this question by comparing rhizosphere diversity in modern maize (*Zea mays*) to its wild progenitor *(Balsas teosinte*). The study concluded that modern maize cultivars have lower Simpson rhizosphere diversity than *teosinte*, indicating that domestication has reduced microbial interactions with plants. On the other hand, Pérez-Jamarillo et al. (2017; 2018) observed no significant difference in α-diversity between domesticated crops of barley (*Hordeum vulgare)*, lettuce *(genus Lactuca)*, and broad bean (*Phaseolus vulgaris*) when compared to their wild progenitors. These mixed results indicate further research is required to fully address this topic.

Despite not finding differences in α-diversity, Pérez-Jamarillo et al. (2017; 2018) found that microbial rhizosphere enrichment differed between wild and domesticated crops. Their 2017 study involved growing wild *Phaseolus vulgaris* and domesticated accessions (all accessions belong to the Colombian Mesoamerican gene pool) in native

Colombian agricultural soil and recording rhizosphere diversity. Through 16s rRNA sequencing, they demonstrated a clear difference in enrichment between the two groups, with wild accessions having a higher relative abundance of *Bacteroidetes* and *Verrucomicrobia* and domesticated accessions being enriched with *Actinobacteria* and *Firmicutes*. To determine whether this difference was a universal trend or specific to common bean, they conducted an enrichment meta-analysis on barley (*Hordeum vulgare)*, lettuce (*genus Lactuca*), and *Arabidopsis*. The meta-analysis revealed results similar to those for common bean, with enrichment of *Bacteroidetes* for wild species and enrichment of *Actinobacteria* and *Proteobacteria* for their domesticated counterparts. In all, these results indicate that this trend may be universal and not merely specific to *Phaseolus*.

The effects of domestication on plant-microbe interactions are believed to be a result of changes to root exudation and root morphology. Root exudates are chemical compounds that a plant releases to manipulate microorganisms in the soil (explained in more detail below). Therefore, changes in root exudation due to domestication may inadvertently affect plant-microbe interactions. Indeed, it has been observed that wild and domesticated plants differ in root exudation (Iannucci et al., 2017; Shaposhnikov et al., 2016). For instance, modern wheat cultivars exude three to five times more "simple sugars" (i.e., fructose, glucose, and maltose) than ancient cultivars (Shaposhnikov et al., 2016). Additionally, modern barley varieties exude a higher amount of sugar alcohols (sorbitol and mannitol) than wild barley (Iannucci et al., 2017). There is a relatively limited amount of research addressing the effects of domestication on root exudation because the collection of root exudates is a complicated task with many obstacles (reviewed in Oburger and Jones, 2018). There currently exists technology to accurately identify and quantify root exudates

through high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GCMS), but resources and methods for effectively collecting root exudates are lacking. If plants are grown in conditions with microbes present, the microbes will metabolize the exudates before collection can occur (reviewed in Oburger and Jones, 2018). However, if the plants are grown in artificial environments with limited microbe presence, such as aeroponics or hydroponics, the collection of root exudates is less difficult, but the applicability of the results is questionable (reviewed in Oburger and Jones, 2018). Until root exudates can be efficiently and effectively collected from natural environments, scientists will be unable to identify the effects of domestication on root exudation.

In addition to root exudation, root morphology, which is linked to plant-microbe interactions, has also been altered by domestication (Martín□Robles et al., 2018; Singh et al., 2019; Szoboszlay et al., 2015). Martín□Robles et al. (2018) compared the root morphology of 30 crop species with their closest wild progenitors and concluded that wild progenitors had thicker and less dense roots, with higher root mass fraction and lower specific root length (SRL). Similar results were obtained in a more comprehensive study of common bean (*Phaseolus vulgaris*), which determined that domesticated accessions primary root lengths are 1.27 times longer than wild accessions (Singh et al., 2019). However, Szoboszlay et al. (2015) obtained contradicting results: *teosinte*, the wild relative of maize, had a larger proportion of fine roots (high SRL), while modern sweet corn root systems had a larger proportion of intermediate-sized roots. However, a limitation to the Martin-Robles et al. (2018) and Szoboszlay et al. (2015) studies is that both used a limited number of wild and domesticated accessions, thus perhaps not fully capturing the genotypic or phenotypic diversity of either group and limiting the applicability of results. Therefore,

further research is required with more comprehensive use of wild and domesticated accessions to fully illuminate the effect of domestication on root morphology.

1.3 Overcoming Challenges in Agricultural Production

The total factor productivity of global agriculture has increased by 72.9% over the past 60 years (USDA, 2019), indicating that over time, humans have become more efficient at farming. However, despite substantial increases in global agricultural productivity, projections still indicate that agricultural production will fail to meet the nutritional needs of future populations (FAO, 2012; FAO 2017; Fischer et al., 2002; Rosegrany and Cline, 2003). The primary obstacles to agricultural productivity meeting global demands are changing climatic conditions (FAO, 2017; Fischer et al., 2002; Rosegrany and Cline, 2003), land scarcity in developing nations (Prosekov and Ivanova, 2018), water scarcity (FAO, 2017; Rosegrany and Cline, 2003), and soil degradation (FAO, 2017). To increase agriculture production and meet the caloric needs of the future world population, the utilization of beneficial soil microbes has been proposed as a sustainable method for increasing food production (Muller and Sachs, 2015; Singh and Trivedi, 2017; Busby et al., 2017). Microbial organisms can increase food production by benefiting crops in a multitude of ways, the most prominent of which are inducing plant growth and facilitating abiotic and biotic stress tolerance (reviewed in Friesen et al., 2011; reviewed in Berendsen et al., 2012; reviewed in Lakshman et al., 2014). The next two subsections summarize how microbes benefit crops and how crops attract beneficial microbes.

1.3.1 Benefits of Microbes

Advances in microbial DNA isolation and reduced costs for deep sequencing have enabled the characterization of the diverse microbial communities that are recruited by crops (Nivelle et al., 2016; Finney et al., 2018). In a field setting, these recruited microbial communities have been shown to increase yields. For instance, increased microbial diversity and activity was correlated with increased yields in potatoes (Larkins et al., 2010), grapes (*Vitis*) (Ingels, Scow, Whisson, & Drenovsky, 2005), cucumbers (Tian, Zhang, Liu, & Gao, 2011), and cotton (*Gossypium hirsutum*) (Mbuthia et al., 2015; Nouri, Lee, Yin, Tyler, & Saxton, 2019). Additionally, the increased presence of beneficial microbes, *Actinobacteria* and *Pseudomona*s, promoted plant growth and yields in grapes (*Vitis*) and peppers (*Capsicum*) under drought stress conditions (Rolli et al., 2014).

In addition to these field experiments, greenhouse and laboratory experiments have also shown that plant-mediated recruited microbial organisms can benefit plants in a multitude of ways (Friesen et al., 2011). A large number of microbes promote plant growth, the most efficient being *Pseudomonas*, *Agrobacterium*, *Bacillus*, and *Azospirillum* (reviewed in Bertrand, Nalin, Bally, & Cleyet-Marel, 2001). Specifically, *Azospirillum brasilense* induces plant growth in common bean (*Phaseolus Vulgaris*) and soybean (*Glycine max*) (Burdman, Kigel, & Okon, 1997), while *Agrobacterium tumefaciens* can promote plant root development (Molla, Shamsuddin, & Saud, 2001).

Along with promoting growth, microbes can also facilitate a plant's ability to respond to biotic and abiotic stresses. For instance, recruited microbes *Pseudomonadaceae*, *Micromonospora*, and *Bacillus subtilis* have the capability to protect plants against

Rhizoctonia solani (root rot), facilitate the plant's ability to sense and respond to drought, and form protective root biofilms, respectively (reviewed in Lakshmanan et al., 2014; reviewed in Bais et al., 2006). Furthermore, *rhizobacteria*, mycorrhizal fungi, and *Trichoderma* can trigger immune responses in plants, protecting them from a broad range of pathogens and herbivores (reviewed in Pineda et al., 2017). Induced immune responses are not only expressed at the site of induction but also systemically and against a broad spectrum of antagonistic organisms in all parts of the plant (Walters et al., 2013; Pieterse et al., 2014).

The two main mechanisms in which immunity is induced in plants is through induced systemic resistance (ISR) and systematic acquired resistance (SAR). These two defense mechanisms work in unison, with ISR being analogous to hypersensitive response and SAR to innate immunity in humans. In ISR, an attack from a pathogen or herbivore triggers microbes to initiate a signal cascade that results in the priming of plant tissue through the jasmonic acid and ethylene pathways (reviewed in Wees & Ent, 2008; Walters et al. 2013). The jasmonic acid pathway activates the expression of defensive compounds while ethylene triggers leaf and fruit abscission, facilitating the removal of infected plant parts (reviewed in Wees & Ent, 2008; Walters et al. 2013). Conversely, in SAR, a pathogen attack triggers microbes to initiate a signal cascade along the salicylic acid pathway, resulting in the production of pathogenesis-related (PR) proteins that act as antimicrobials and inhibitors of virulence factors (Pieterse et al., 2014). The utilization of different pathways by ISR and SAR increases plant protection against pathogens that develop resistance to either immune response (Choudhary & Prakash, 2007).

1.3.2 Recruiting Beneficial Microbes through Root Exudates

Plants manipulate microbial communities through the secretion of root exudates, or "info-chemicals," composed of secondary metabolites (flavonoids, organic acids, amino acids, etc.), mucilage, enzymes, ions, and free oxygen (reviewed in Bais et al., 2006). Root exudation is carbon costly to the plant, with about 20% to 80% of photosynthates being exuded (reviewed in Saleem et al., 2018). However, the costly nature of root exudation is offset by the numerous benefits the plant receives in return, such as attracting beneficial microbes, repelling antagonistic microbes, allelopathy, and altering environmental conditions (changes to soil chemistry, increasing micronutrients, etc.) (reviewed in Bais et al., 2006).

The roles of specific root exudates in plant-microbe interactions remain largely unknown (Jacoby et al., 2017; Sasse et al., 2018). However, a few studies have identified specific classes of compounds that are crucial for plant-microbe interactions. For instance, legumes are the only plant family to produce isoflavonoids, which play a major role in the attraction, identification, and colonization of nitrogen-fixing rhizobia (reviewed in Bais et al., 2006). Additionally, the exudation of flavonoids by a broad group of plants are believed to be responsible for facilitating the growth of arbuscular mycorrhizal fungi, a fungus which helps plants acquire limiting nutrients like phosphorus (reviewed in Bais et al., 2006).

1.4 The Study of Domestication can Facilitate Agricultural Production

Agricultural production faces many difficulties in ensuring global food security. A potential sustainable method for overcoming these hurdles is through the selection of

belowground traits, such as increased beneficial-microbe interactions or altered root systems with more adaptive phenotypes that allow crops to better tolerate abiotic and biotic stressors. However, research on belowground traits has been limited due to inadequate noninvasive methods for quantifying and categorizing root traits. Nevertheless, recent advances in phenotyping have afforded us the opportunity to address some of these issues and potentially increase agricultural production (Tracy et al., 2019). Furthermore, the study of domestication and crop wild relatives could be a valuable resource in the attempt to increase agricultural production. Crop wild relatives and landraces have been a key source for genetic and phenotypic variation in crop improvement efforts (Warschefsky et al. 2014). In chickpea, for instance, it has been estimated that 93.5% to 97.5% of progenitor genetic variation is absent from breeding programs, highlighting the repository of novel variation in crop wild relatives (von Wettberg et al., 2018). Therefore, by utilizing wild relatives and recently developed phenotyping technologies, we can further our understanding of how these agronomically important traits have been inadvertently affected by domestication, which would then allow us to potentially identify and reintegrate lost traits from crop wild relatives to facilitate efforts to improve agricultural production.

1.5 Dissertation Outline

I begin my dissertation with Chapter 1, a review of the effects of domestication on domesticated plants and, more specifically, the recent research that has categorized the effects of domestication on belowground traits like root morphology and plant-microbe interactions. Within this chapter, I also discuss the obstacles that agricultural production currently faces, including an ever-growing human population and climate change, and conclude with how the study of domestication and crop wild relatives may provide

potential solutions to these challenges. The purpose of this chapter is to create a foundational level of knowledge about domestication and agricultural production, so readers become familiar with the questions I address in the following chapters.

Chapter 2 presents data focused on the economic and nutritional value of the "lost" stay-green trait in chickpea. Increasing the economic values of crops is a well-established practice in the breeding community. For instance, breeders have increased the sugar content in corn and created "supersweet" hybrid varieties with increased palatability, longer shelf life, and higher quality of both fresh and processed corn products (ERS, 2007; Hansen, 2019). These resulting phenotypes have helped increase U.S. corn sales by 32.4% from 2012 to 2017 (USDA, 2017). Increasing the economic value of crops is imperative in developing nations, where the majority of agricultural production is conducted by small-holder farmers who are impoverished or are on the brink of poverty (FAO, IFAD, UNICEF, WFP, & WHO, 2017) The state of food security and nutrition in the world. Building resilience for peace and food security.). For such farmers, maximizing the profits of their crops is essential in lessening financial burdens.

In Chapter 2, we address economic value in chickpea, one of the world's most important dietary legumes (FAOSTAT, 2019). Chickpea is primarily grown and consumed in developing countries like India, Turkey, and Ethiopia (FAO, IFAD, UNICEF, WFP, & WHO, 2017). The state of food security and nutrition in the world. Building resilience for peace and food security.), where small-holder farmers sell fresh green chickpeas to maximize their profits. Fresh green chickpeas are sold at premium value over traditionally dried chickpeas because they are only available for about three months of the year.

Recently, we discovered a new green-seeded phenotype of chickpea that may have been lost during domestication and could potentially allow small-holder farmers to sell chickpeas at a premium for a longer duration. In this chapter, we first identify the molecular mechanisms of green seediness—a convergent phenotype that is found in many other domesticated legumes such as peas, fava bean, and soybean. Additionally, we identify whether this phenotype is categorized as a functional or cosmetic stay-green trait by measuring its physiological responses to drought conditions, a common environment experienced by chickpea grown in the semi-arid tropics. Lastly, we measure the provitaminogenic carotenoids (the precursors to vitamins) present in the seeds to analyze this phenotype for its nutritional properties, as green-seeded legumes have been observed to have higher nutritional properties than other color seeds. Therefore, in addition to increasing chickpeas' economic value, this stay-green trait may also simultaneously increase its nutritional value.

Chapter 3 presents further data addressing a major limitation in domestication studies, the use of a limited number of accessions of crop wild relatives (CRW). Research has demonstrated that CRWs have great genetic diversity and are a source of beneficial traits in crop improvement programs (Warschefsky, 2018; explained in more detail above). Despite these features, domestication studies still only use a handful of CRW accessions to represent the entirety of the CRW community. I address this issue by taking a whole-plant approach and assessing the response (below- and aboveground traits) of chickpea to a novel environment (with/without nitrogen), through the use of modern cultivars and CWR accessions systematically collected from chickpea's native range (von Wettberg et al., 2018). Furthermore, the wild accessions used in the study represent the entire genetic

diversity of the chickpea's closest compatible wild relative, *Cicer reticulatum* (von Wettberg et al., 2018). Lastly, by measuring chickpea's response to a novel environment, we can assess whether domestication has hindered its ability to respond to new environments, an important factor in overcoming some of the current problems hindering agricultural production.

Chapter 4 discusses current opinions on how to measure and improve agricultural production through the use of cover crops and intercrops. In this chapter, we first discuss the limitations in current cover cropping and intercropping studies and discuss how a plant-soil feedback approach—an approach most commonly used in natural systems—can be easily adapted to agricultural systems to potentially bolster agricultural production. We then coin and define the terms "rotational value" and "intercropping value" and provide a mathematical formula to quantify the rotational and/or intercropping value of a crop. We then explain the potential benefits and limitations of using these terms and formulas and provide methods for increasing these values. For instance, rotational and intercropping values could be increased by breeding for enhanced ecosystem services such as weed suppression and beneficial microbe recruitment. By improving these ecosystem services, the crop should theoretically improve as a rotational or intercropping partner, thus increasing agricultural production.

Chapter 5 presents an empirical exploration of the rotational value concept from Chapter 4. Through a field experiment, we test for differential rotational values present within field pea. In this study, we use a mixture of modern cultivars, early landraces, and wild accessions, and measure how each accession affects soil properties such as nutrient

mobilization and microbial community and the growth and yield of a subsequently grown crop. The main purpose of this chapter is to illustrate the existence of differences between accessions for cover cropping traits, demonstrating that breeding for better rotational partners is feasible. The incorporation of landraces and crop wild relatives is also a novel concept, as these groups are typically underutilized in cover cropping research. As previously mentioned, these groups have also been shown to have higher genetic diversity and are a source of beneficial traits for crop improvement efforts (Warschefsky, 2018; explained in more detail above).

Lastly, Appendix A provides data identifying cover cropping pea cultivars that are suitable for overwintering in Vermont. Overwintering peas in Vermont is difficult due to the lack of constant snow cover that would act as an insulator to protect the peas from harsh winter temperatures. As a result of the highly variable snow cover in Vermont, overwintering peas have yet to become an established farming practice despite its agricultural and ecological benefits. Despite these limitations, we contacted collaborators from the USDA in Pullman, Washington, and received plant material that may be suitable for Vermont winters. We conducted a winter trial to test the viability of these cultivars as overwintering cover cropping field pea. The results presented in this appendix will hopefully help bolster local agricultural production.

1.5.1 Intellectual Merit

The research presented in this dissertation advances our understanding of domestication in numerous ways: we identify the molecular and phenotypic mechanisms of an agronomically important domesticated convergent trait, assess the effect of

domestication on crops in adapting to novel conditions, and assess the effect of domestication on agronomically important cover cropping traits such as nutrient mobilization and microbial recruitment. Furthermore, in addition to the basic question we address regarding domestication, a portion of my research also has an applied aspect that directly contributes to helping farmers increase yields and/or profits. Lastly, the information presented in this dissertation will inform a wide variety of audiences, from farmers and crop management personnel to breeding programs and agronomic scientists. As a whole, my dissertation advances efforts to bolster agricultural production in the face of changing climatic conditions to meet the nutritional demands of an ever-growing human population.

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CHAPTER 2: FUNCTIONAL DISSECTION OF THE CHICKPEA (CICER ARIETINUM L.) STAY-GREEN PHENOTYPE ASSOCIATED WITH MOLECULAR VARIATION AT AN ORTHOLOG OF MENDEL'S I GENE FOR COTYLEDON COLOR: IMPLICATIONS FOR CROP PRODUCTION AND CAROTENOID BIOFORTIFICATION

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2.2 Abstract

"Stay-green" crop phenotypes have been shown to impact drought tolerance and nutritional content of several crops. We aimed to genetically describe and functionally dissect the particular stay-green phenomenon found in chickpeas with a green cotyledon color of mature dry seed and investigate its potential use for improvement of chickpea environmental adaptations and nutritional value. We examined 40 stay-green accessions and a set of 29 BC2F4-5 stay-green introgression lines using a stay-green donor parent ICC 16340 and two Indian elite cultivars (KAK2, JGK1) as recurrent parents. Genetic studies of segregating populations indicated that the green cotyledon trait is controlled by a single recessive gene that is invariantly associated with the delayed degreening (extended chlorophyll retention). We found that the chickpea ortholog of Mendel's I locus of garden pea, encoding a SGR protein as very likely to underlie the persistently green cotyledon color phenotype of chickpea. Further sequence characterization of this chickpea ortholog CaStGR1 (CaStGR1, for carietinum stay-green gene 1) revealed the presence of five different molecular variants (alleles), each of which is likely a loss-of-function of the chickpea protein (CaStGR1) involved in chlorophyll catabolism. We tested the wild type and green cotyledon lines for components of adaptations to dry environments and traits linked to agronomic performance in different experimental systems and different levels of water availability. We found that the plant processes linked to disrupted CaStGR1 gene did not functionality affect transpiration efficiency or water usage. Photosynthetic pigments in grains, including provitaminogenic carotenoids important for human nutrition, were 2–3-fold higher in the stay-green type. Agronomic performance did not appear to be correlated with the presence/absence of the stay-green allele. We conclude that allelic variation in

chickpea CaStGR1 does not compromise traits linked to environmental adaptation and agronomic performance, and is a promising genetic technology for biofortification of provitaminogenic carotenoids in chickpea.

2.3 Introduction

chickpea is an important source of nutrition and economic livelihood for developing countries [1]. In developing semiarid tropical (SAT) regions, chickpea is typically grown during the post-rainy season under rain-fed conditions [2]. As a result of this growing practice, fluctuations in crop yields largely reflect in-season water availability and crop adaptation to these conditions. Fluctuations in crop production threaten the nutritional and economic status of the already impoverished smallholder farming communities, which make up 80% of all Asian and African farmers [3]. One way to alleviate chickpea production fluctuations in SAT is through the introduction of cultivars with enhanced climate resilience and nutrient density. The utilization of functional stay-green phenotypes is a possible solution to enhance crops climate resilience due to its ability to conserve water and nutrients in drought conditions [4]. Functional stay-green technology is extensively studied and exploited by many crop improvement programs (mainly in cereals, sorghum: [5–10]; maize: [11–14]; wheat: [15–19]; rice: [20–23].

The biological basis (i.e., plant constitutive water and nutrient use dynamics) and benefits of the functional stay-green trait for the SAT agrisystems have been well documented [10,24–28]. In contrast, cosmetic-stay green which is linked to naturally occurring loss-of-function allelic variants [29] with dysfunctional chlorophyll degradation pathways, has rarely been studied in these conditions. This type of stay-green results in

extended retention of chlorophyll in all plant organs (leaves, stems, grains) and delays age-related senescence as well as senescence caused by environmental factors (e.g., drought). The utility of cosmetic stay-green variants has been, thus far, limited to green color retention in ornamentals, vegetables, and turf-grasses [29]. However, green-seeded variants also occur in many legumes and pulses such as, chickpea, common bean, lima bean, lentil, cowpea, and pea. Seed greenness in pea has resulted into two major market categories, yellow and green pea, demonstrating the vast economic potential of this trait in other legumes and pulses.

The cosmetic stay-green trait might have much more practical implications than just visual appearance caused by extended chlorophyll retention [29–33]. For example, it is well known that chlorophyll biosynthesis and retention is co-regulated with carotenoids which facilitate scavenging of reactive oxygen species generated in the process of photon's capture by chlorophylls [34–36]. Therefore, we may expect that extended chlorophyll maintenance in any plant organ (including seeds) to be associated with extended maintenance of carotenoids (including β-carotene, i.e., provitamin A), which are of relevance to improving the human diet [37,38] as observed in chickpea [30,39]. On the other hand, the retention of chlorophyll and its associated pathways in cosmetic stay-green crops may impose drawbacks on crop agronomic performance, such as slow seedling establishment or arrested N-remobilization [29,40–47].

Therefore, in this study we aim to characterize the genetic, molecular and physiological basis of cosmetic stay-green trait in chickpea. We document allelic variation in the chickpea ortholog of the 'staygreen' protein that is invariantly associated with genotypes of the green cotyledon color. We test the functional consequences of 'stay-

green' on several key plant processes linked to water usage, transpiration efficiency, and other agronomic traits important for chickpea production in drought-prone regions of the semiarid tropical (SAT) agrisystems. Lastly, we examined stay-green's potential for natural biofortification of chickpea to alleviate the nutritional deficiencies commonly found in these systems.

2.4 Methods and Materials

2.4.1 Plant Material: Chickpea Germplasm and Breeding Lines

Chickpea genotypes with the common yellow cotyledon color and those with the infrequently occurring green cotyledon color were obtained from gene banks (USDA GRIN in Pullman, Washington, and ICRISAT India) and from chickpea improvement programs (detailed in Supplementary Table S1). In the process of plant grow outs for seed multiplication, the gene bank accessions were visually screened for occurrence and confirmation of green cotyledon color in mature dry seeds. Furthermore, during such grow outs we examined degreening of leaves of this germplasm accessions using a detached leaf assay, wherein leaves were wrapped in aluminum foil (to block out light and trigger degreening) and the pigment loss/retention capacity ("degreening") assayed 5–10 days later. The same plants were tested for sequence polymorphism in the CaStGR1 gene (see below). In initial germplasm screen, eight lines with green cotyledon color representing four different allelic variants in the chickpea stay green candidate gene and two yellow cotyledon genotypes carrying the wild-type alleles were used in physiological studies (Supplementary Table S1). In these initial studies, as expected [30] elevated levels of total carotenoids among green cotyledon color lines relative to concurrently grown normal yellow chickpea lines was observed.

For the subsequent and more detailed analyses, breeding lines with contrasting yellow and green cotyledon color were used (Supplementary Table S1). These lines were derived from introgression of the green cotyledon trait from the germplasm accession ICC 16,340 into two Indian elite chickpea cultivars, JGK1 and KAK2 with yellow cotyledon colors. 25 BC4-5:F2 generation introgression lines and their parents were screened for phenology and agronomic traits. Based on homogeneous phenology (flowering time, duration of flowering) and agronomical traits (harvest index), genotypes were selected for further studies (Supplementary Table S3) details of genotypes used in different experiments).

2.4.2 Molecular Characterization of Candidate Gene and Genome:

The genotypes tested for variation in CaStGR1 allele are shown in S1 table. In these, the genomic DNA was extracted from the young leaflets using QIAGEN DNeasy Plant Kit following the manufacturer's recommended procedures, or from seed-derived cotyledon tissue (for the cultivar 'CDC Verano') using a phenol-chloroform based extraction protocol. PCR amplification for CaStGR1 were performed with ExTaq polymerase (Takara-Fisher) using oligonucleotide primers as detailed in Supplementary Table S2. PCR products were analyzed in 1% agarose gel electrophoresis. For Sanger sequencing, PCR amplicons were purified with ExoSAP kit (Affymetrix, Santa Clara, CA, USA) to remove any excess salts carried over from PCR reactions. Amplicons were Sanger sequenced using single primers at on-campus core sequencing facilities at the University of California and the University of Vermont. Chromatogram traces from amplicon sequencing were analyzed with the Sequencher (Gene Codes Corporation, Ann Arbor, MI

USA) and Geneious 2019.1 software packages. Sanger sequence traces were curated manually to identify and verify the positions of variant nucleotides in sequencing data. Variants supported by at least two independently run sequencing reaction were recorded and used for enumerating allele distribution and frequencies.

Preparation of whole genomic libraries for Illumina sequencing and data analysis of Illumina short read data were as described previously [65]. Illumina reads were mapped to the *C. arietinum* 'CDC Frontier' reference genome assembly [50] using BWA MEM 0.7.9a-r786. Visualization of CaStGR1 and its flanking regions was done using an instance of GBrowse loaded with gene structural annotation available from the CDC Frontier reference.

For genotyping of the CaStGR1-1 allele as a CAPS marker, PCR products were digested with Hpy-188I restriction enzyme (New England Biolabs, USA) per manufacturer's recommended protocol. Digested PCR products were analyzed by gel electrophoresis in 1.35% agarose gels in 0.5× Tris Borate EDTA buffer stained with cybersafe reagent. Genotyping of the CaStGR1-4 allele in F2 population of wild type (yellow cotyledon) genotypes and green-cotyledon lines was conducted as a customized KASP assay (LGC Genomics, UK) using leaf tissue from greenhouse grown plants and oligos listed in Supplementary File S2.

2.4.3 Plant Growth Conditions for Physiological Assays (Experiments Listed in Table 3)

2.4.3.1. Experiments Conducted in Glass-House (Experiment 1, 2, 3a and b)

The glass-house environment was used to evaluate crop responsiveness to soil and atmospheric drought. In Experiments 1 and 2 (Supplementary Table S4), plants were grown in 8" plastic pots filled with 5 kg of vertisol while for experiment 3a and b, plants were raised in PVC cylinders filled with 45 kg of vertisol. The experiments were set-up using completely randomized block design with treatments as separate blocks. The black soil (Vertisol) was collected from the ICRISAT farm and fertilized with DAP (di-ammonium phosphate) at the rate of 0.3 g per kg of soil in all experiments. Seeds were treated with fungicides (Thiram®; Sudhama Chemicals Pvt. Ltd. Gujarat, India) to avoid fungal contamination. Four seeds were sown in each pot, and a rhizobium inoculum (Strain No: IC 2002) was added to each pots to ensure adequate nodulation. Two weeks after sowing, plants were thinned to two plants per pot. Plants were maintained well-watered up to ~30 days after sowing. During the experiments duration, a data logger (Lascar Electronics Inc. Whiteparish, United Kingdom) was positioned within the plant canopy for the hourly recording of the air temperature and relative humidity (RH%) and these oscillated on average between 28–22 °C and 70–90% during the day–night cycle.

2.4.3.2. Experiments Conducted at LeasyField (Experiment 3c)

The Lysimetric facility located at International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) Patancheru in India (17°30'N; 78°16'E; altitude 549 m). It offers an experimental setup to evaluate the basic crop agronomic features, monitor the crop capacity to convert water into biomass (g of dry mass per unit of water transpired) and to measure water use patterns during the cropping season. Plants were grown in lysimeters constructed from the PVC plumbing pipes with 20 cm diameter and 1.2 m length outdoors under a rain-out shelter (ROS) (Experiment 3c). The protocol for lysimeter soil

preparation & filling, spacing arrangement, growing and weighing plants were followed according to [69,70] and [60,77]. Three seeds were sown in each cylinder and watered regularly and around 15 DAS thinned to one seedling per cylinder. The experiment was planned in a complete randomized block design. One block was assigned to a well-watered treatment (WW) and two blocks to water-stressed treatment (WS). The WS treatment was imposed by cessation of watering from 25 Days after sowing (DAS). WW plants were watered every week to maintain 80% field capacity until maturity. During the experiment's duration, the data logger was positioned within the plant canopy to record the day and night temperatures and relative humidity (RH%), which fluctuated under the natural day–night oscillations around average 31.7/15.5 °C and 40/85%.

2.4.3.3. Experiments Conducted at LeasyScan (Experiment 4)

LeasyScan is a high throughput phenotyping platform constructed to monitor crop canopy related parameters during the vegetative phase of development with high throughput and accuracy. Details of LeasyScan technology and set-up are elaborated in [61,62,64]. For experiment 4 the crop was raised in large trays (60 × 40cm, approximately 75 kg of vertisol; i.e., "miniplots") filled with vertisol using the recommended field management practices (20 $kg \cdot ha^{-1}$ of DAP and planting densities of 32 plants m^{-2}). The experimental design was an Alpha lattice with 4 replications to account for spatial variability. Plants were maintained under well water conditions throughout the experiment. Canopy size related parameters (i.e., 3D-Leaf area, digital biomass and leaf area index) were continuously measured from 15-40 DAS when the plants were harvested. During the crop grown period the daily temperature and humidity oscillated in between of 11/35.8 °C

and 17.2/93.2% on average as per the records of the attached weather station (Model: WxPRO™; Campbell Scientific Ltd., Shepshed, United Kingdom).

2.4.3.4. Experiments Conducted in Field (Experiment 5)

The main crop agronomic features were measured in the field experiment that was planted in post-rainy 2017–18 season at ICRISAT field facilities. The field was solarized using a polyethene mulch during the preceding summer primarily to avoid the crop infection by *Fusarium oxysporum* f. sp, [78]. The basal dose of di-ammonium phosphate at the rate of 18kg N ha^{-1} and 20kg P ha^{-1} was applied before sowing. The field was prepared as broad bed and furrows with 1.2m wide beds flanked by 0.3m furrows. Within these beds, the plots of 4 rows of 4 m length were planted. Seeds were treated with Thiram® (Sudhama Chemicals Pvt. Ltd. Gujarat, India) to avoid fungal contamination during germination. The seeds were hand sown at a depth of 2–3 cm maintaining a row-to-row distance of 30 cm and a plant to plant distance of 10 cm (i.e., 33 plants m^{-2}). After sowing, furrow irrigation (60 mm) was given to ensure uniform seedling emergence. Subsequently, plants were grown under different irrigation regimes: water stress [WS; crop received only ~60 mm at the sowing], and well water [WW; crop received ~60mm at the sowing and additional ~20 mm irrigation every 20 days through perforated irrigation system]. The plots were kept weed-free by hand weeding and intensive protection was taken against pod borer (*Helicoverpa armigera*). The experiment was conducted in a randomized complete block design with three replications for each treatment (WW/WS).

2.4.4 Physiological Assays

2.4.4.1. Experiments to Test Plant Responsiveness to Soil Drought (Experiment 1b and 1c)

The main aim of "dry-down" experiments is to assess the capacity of genotypes to restrict the transpiration upon declining soil moisture, which could be a crucial adaptive trait for plants in particular water-limited environments. To test the transpiration restriction capacity of selected genotypes, these were organized in two experimental blocks; well-watered (WW) and water-stressed (WS) conditions. The day before the dry-down was initiated all pots were abundantly watered and the soil was allowed to drain overnight. The following day the soil surface of the pots were covered with plastic sheets, and then a uniform 2 cm layer of plastic beads to prevent soil evaporation. The pots were then weighed and this initial pot weight was considered as the soil-saturation level (field capacity) of the individual pots. Pot weight was recorded daily at the same time of day. Based on the daily weight loss, the well-watered plants were maintained at approximately 80% of the saturated weight (80% of the field capacity). For the WS treatment, the water available to the plant was gradually decreased by allowing a maximum daily water loss of 70g. The transpiration weight loss above 70g was compensated by adding an excess amount of transpired water to each pot. The experiment was terminated when transpiration of all WS plants was below 10% of their WW treated counterparts. After termination, the above-ground biomass of the plants was harvested, organs separated, and oven-dried at 60 °C for a minimum of 3 days. The traits assessed are detailed in Supplementary Table S4.

Additionally, during the dry-down experiments (in Experiment 1b and 1c), 30 mg leaf tissue (leaflets from the first fully developed leaf from the top of the main stem) from each replicate (i.e., in WW and WS) were collected twice WW and severe water stress (~0.25 NTR). Collected tissues were frozen by liquid nitrogen and conserved for later estimation of pigments (i.e. Chlorophylls and Carotenoids, see below).

(http://gems.icrisat.org/allinstruments/controlled-imposition-of-water-stress/; methodology also used in e.g., [79–81])

2.4.4.2. Experiments to Test Plant Responsiveness to Atmospheric Drought (Experiment 2a,b,c)

While "dry-down" experiments (above, experiment 1b and c) were conducted to evaluate plant responsiveness to drying soil, complementary "transpiration responsiveness" experiments were designed to characterize the genotypic ability to limit transpiration upon drying atmosphere [increasing vapour pressure deficit (VPD)]. For this, the plants were evaluated during vegetative growth stage under well-watered conditions. Around 30-day-old plants grown in pots were watered to ~90% field capacity and soil evaporation minimized by applying the plastic sheets and beads similarly as in the regulated dry down experiment (above). Initially, the plant transpiration was evaluated outdoors during the cloud-less clear days in the natural circadian cycle or in the growth chambers (Conviron-PGW36 model, Controlled Environments Limited, Winnipeg Manitoba, Canada: see more details in http://www.conviron.com/sites/default/files/PGW36%20Data%20Sheet_1.pdf). In these experiments, temperature and humidity sensors were mounted at canopy level to record the actual conditions experienced by the crop canopy in 5 min intervals. In the outdoors conditions, plants were weighted in hourly intervals using 0.01 g precision scales (KERN 24100, Kern & Sohn GmbH, Balingen, Germany). Consequently, for the controlled environment testing, the same pots were placed into the growth chamber for one day to acclimate with the day/night temperature (°C) and relative humidity (RH%) of 32/26 °C and 60/80% respectively. Plants were then exposed to an increasing ladder of VPD ranging

from 0.9 to 4.1 kPa by increasing temperature and decreasing RH% (80–30%) at hourly intervals for 8 h. Plant transpiration was also assessed hourly by swift weighing in between of the VPD transitioning regimes. At the end of the experiments, plants were harvested and leaf area (LA) was measured with a leaf area meter (LI-3100C area meter, LI-COR®Biosciences, Lincoln, Nebraska, USA). Consequently, the plant transpiration rate was expressed as TR = T/LA [g of water transpired per unit of LA per hour] and regressed upon VPD during the particular time interval. In both germplasm and ILs (Experiment 2a and 2c), the specific leaf weight (SLW) was estimated as leaf dry weight (g)/leaf area (cm^{-2}).

(http://gems.icrisat.org/allinstruments/transpiration-response-to-increasing-vpd/; methodology also used in e.g., [61,62,79,80])

2.4.4.3. Experiments to Test Plant Baseline Agronomic Features and Water-Use Related Traits in Lysimetric Facility (Experiment 3a,b,c)

The unique lysimetric set-up allows estimating the plant water productivity while having access to relevant agronomic traits. The cylinders were covered with plastic sheets and beads similarly as in assay #1 and 2 and the water use monitoring started ~25 DAS. From this onwards, the cylinders were weighed weekly by lifting them with a block chained pulley using S-type load cell (Mettler-Toledo, CSE 100, Geneva, Switzerland) until crop maturity. The WW block of experimental plants was retained at 80% of field capacity. Under the WS treatment, the declining soil moisture was only monitored but not regulated, which contrasts with the regulated dry-down protocol used in the pot culture (see above #1). During the plant growth flowering dates were recorded for each plant. At the end of

the experiment, plants were harvested, the crop residuals dried at 60 °C in an oven during minimum 72 h and the above ground biomass, grain and vegetative dry biomass were weighed (KERN 3600 g; 0.01 g precision balance, Kern & Sohn GmbH, Balingen, Germany). Plant transpiration was calculated from consecutive cylinder weight differences and water additions. Transpiration efficiency (TE; [gram of biomass per kilogram of water transpired; g/kg^{-1}]) and water use efficiency (WUE, [gram of seed weight per kilogram of water transpired; g/kg^{-1}]) was then calculated as the ratio of the total/grain dry biomass per unit of water transpired. Lastly, Harvest Index (HI) was calculated as the ratio of total dry grain biomass per the total dry weight of remaining above-ground biomass. (http://gems.icrisat.org/allinstruments/lysimetric-assessments/, methodology also used in e.g., [60,69,70,82,83]).

2.4.4.4. Experiments to Assess Plant Canopy at LeasyScan (Experiment 4)

The LeasyScan platform has been used to monitor traits indicating crop canopy traits related to "vigor". This is enabled by the optical system (PlantEye®; www.phenospex.com), which captures the dynamics of canopy growth during the crop vegetative growth-phase with high throughput and accuracy. We measured 3D-Leaf area (3D-L; canopy size reconstructed from 3D point-cloud distribution [mm^3]), projected leaf area (PL; canopy ground coverage [mm^2]) and plant height (PH; estimated from 3D point-cloud as height encompassing 95% of recorded points of given point-cloud) during 15-30 DAS (http://gems.icrisat.org/leasyscan/) methodology also used in e.g., [4,61,64]).

2.4.4.5. Agronomic Evaluation of ILs in Field Settings (Experiment 5)

Agronomic traits of selected stay-green introgression lines and their recurrent parents were evaluated using the precision field facility under optimal water input (WW) and under severe water shortage WS. Under both treatments, in each plot we monitored the phenology parameters (date to first flower, 50% flowering and 80% of the dried pods was recorded as maturity). At maturity, shoots were harvested plot wise and kept for drying at 60 °C for minimum of 3 days. Organs were separated, dry weights recorded and expressed in grams per meter square (g m^{-2}). 100 seed number was counted by seed counter (Data Count S60 seed Counter, Data technologies, Israel; http://www.data technologies.com/data_count_s_60_seed_counter.html), weighed and based on these the total seed number per square meter was calculated.

Harvest index was calculated:

(1)

HI = (Seed weight/total shoot biomass weight) × 100 [%].

2.4.5 Chlorophyll and Carotenoid Estimation in Leaves and Seeds (Measured in Experiment 1 and 3)

Photosynthetic pigment contents (chlorophyll a, chlorophyll b and total carotenoids) were assessed in the leaf tissues across various stages of plant exposure to declining soil content in lysimeters (un-regulated dry-down; Experiment 3) and in pot cultures (regulated dry-down; Experiment 1b and 1c). The grain pigments were assessed only in the experiments conducted at lysimetric experiments (Experiment 3a,b,c).

In Experiment 3c the leaf tissue samples were collected from each plant from the glasshouse lysimetric experiment. Chlorophyll a and b, as well as Carotenoids, were

estimated from the samples using dimethyl sulfoxide (DMSO) method [84]. We standardized that around 18 mg of fresh leaf tissue/30mg of dry-seed powder extracted in a 5mL of DMSO resulted in suitable optical density (OD) between 0.3–0.9. The test-tubes with the exact weighted tissue and DMSO were placed in ~65 °C hot water bath and left for cca 3 h until the tissue became translucent ensuring all pigments were extracted into to the DMSO. The OD of extract was assessed spectrophotometrically (Shimadzu UV-2401 PC UV-Visible Spectrometer; Shimadzu Scientific Instruments) at 665.1 (Chlorophyll A), 649.1 (Chlorophyll B) and 480 (Total Carotenoids) and the contents were calculated as per [84].

The grain material from Experiment 3b was used to separate the main carotenoids using the High Performance Liquid Chromatography (HPLC) system. For this, the extraction of carotenoids was done according to the method of [85] with some modifications. Briefly, about 0.1 g of chickpea sample was weighed and placed in a screw-capped glass tube (~15 mL tube) and 1 mL ethanol containing 0.1% butylated hydroxytoluene (BHT) added to the solution. The mixture was saponified by adding 200 µL of 20% Potassium hydroxide (KOH) and mixed by vortexing. Extraction was completed by adding 1.5 mL hexane to the saponified solution, vortexed for 20 s and centrifuged at 2500 rpm for 5 min. Using a glass pipette, the upper hexane layer containing carotenoids was carefully removed and transferred to a new glass tube. Extraction was repeated 2 more times. The combined hexane extracts were then dried down under a stream of nitrogen gas. Purified β-apo-8′-carotenal was used (absorbance ~ 0.8; 100 µL) was used as an internal standard. The dried extract was reconstituted in 100 µL of 50:50 (v/v)

methanol:dichloroethane and 10 µL of the sample injected into the HPLC system (duplicate injections per sample).

Chromatographic separation of carotenoids was carried out using the Ultra-Fast Prominence Liquid chromatography (Shimadzu, Kyoto, Japan) equipped with a SIL-20ac-xr Prominence auto-sampler, a DGU-20A5 Prominence degasser, a CTO-20AC column oven and an SPD-M20A Diode Array Detector (DAD). Separation of carotenoids was achieved at 25 °C on a C30 YMC carotenoid column (250 × 4.6 mm, i.d., 5 µm particle size, Waters, Ireland) on a gradient method with 95% Methanol as solvent A and 100% MTBE as solvent B. Identification of the carotenoids was based on the standards, their retention times and by comparing the absorption spectra with those in the literature. Quantification of the carotenoids were extrapolated from standard curves prepared from authentic standards after correcting for extraction efficiency based on the recovery of the internal standard. The processing of all chromatograms was done using Shimadzu LC Lab-Solutions software (also used in [26,84,85]).

2.4.6 Statistical Analysis

In the experiments 1b, 1c, 2a, 2c, 3a, 3b, 3c, 4 and 5, the differences between investigated genotypes were evaluated by simple/multiple-way ANOVA followed by the Tukey–Kramer test to evaluate the significance of genotypic differences (Statistical program package CoStat version 6.204 (Cohort Software, Monterey, CA, USA). The line graph (Experiment 2a, 4), bar graph (1b, 1c, 2a, 2c, 3a, 3b, 3c, 4 and 5) and simple linear regressions were fitted using Microsoft Excel 2013 (Microsoft Corp., Redmond, Washington, USA). For treatment of temporal data from experiments 1b, 1c and

experiments 2 a,c-i.e., transpiration response to atmospheric (Experiment 2a and 2c) and soil drought (Experiment 1b and 1c) we used methodologies described in [69,70,80,86,87]; specifically, a nonlinear regression analysis was done using GraphPad Prism version 6 (GraphPad Software Inc., San Diego, California, USA), and Genstat 14.0 (VSN International Ltd., Hemel Hempstead, UK).

2.5 Results

2.5.1 Delayed Degreening Phenotypes in Green Cotyledon Chickpea and Underlying Allelic Variation

2.5.1.1. Delayed Degreening Phenotypes in Green Cotyledon Chickpea

In the initial examination of two green-seeded accessions, PI 450,727 and W6 25975, we observed a delay in degreening of mature plant tissues after harvesting, including of leaves and pods. Subsequent senescence assays of fresh growing leaves (Figure 1) corroborated the initial observations of delayed degreening that were made on harvested whole plants.

To determine the extent of co-occurrence of delayed degreening of leaf tissues and the green cotyledon trait, we examined degreening in a broader set of green cotyledon chickpea. Using the detached leaf assay, examined degreening among 30 green-seeded chickpea germplasm available from the public gene banks, alongside four other germplasm lines with yellow cotyledon color (Table S1). In this experiment all 27 green cotyledon accessions (three other accessions did not germinate) exhibited delayed degreening, with detached leaves remaining remained visually green through day 7 of the detached leaf assay (Table S1). By contrast, each of the four yellow cotyledon accessions exhibited an

apparently normal degreening phenotype, with progressive yellowing of leaves clearly evident by day 7 after the start of the experiment (Figure 1c,d). Furthermore, in a separate experiment, we examined degreening of leaves of this germplasm accessions using an "on-planta" assay, wherein leaves were wrapped in aluminum foil (to block out light and trigger degreening) and degreening assayed 5–10 days later (Figure 1e,f). Of 29 accessions assayed in this manner, all 26 green cotyledon lines exhibited persistent green leaves, while by contract, the three yellow cotyledon accessions exhibited yellow-colored leaves (Table S1). Together, the data from the two different assays for degreening invariantly correlated green cotyledon seed types with delayed degreening (senescence) of leaf tissues, and which contrasted with a more rapid (normal) senescence of leaves of the yellow cotyledon seed types. Moreover, this association held true in additional genotypes (breeding lines or cultivars) that were analyzed subsequently (Supplementary Table S1).

2.5.1.2. Identification of Chickpea Ortholog of the Staygreen (SGR) Protein

The delayed degreening observed to be associated with the green cotyledon colored chickpea was reminiscent of the 'stay green' phenotype. This suggested that the 'staygreen' gene as a potential candidate gene in chickpea, as this protein has been previously shown to underlie the green-cotyledon trait at Mendel's I locus in garden pea [48,49]. To identify chickpea sequence homologs of SGR protein, coding regions of SGR protein from pea and Medicago [33] were used in blast searches to identify chickpea transcript assemblies and genomic sequences from public databases. Alignment of messenger RNA sequences against the genomic sequence of chickpea indicated a gene structure comprised of four exons interspersed with three introns (Figure 2a and Supplementary Figure S1). Oligonucleotide primers were designed to encompass the entire coding region of the STG

gene and used for PCR amplification from cDNA and genomic DNAs of yellow cotyledon chickpea. Amplified PCR products were Sanger sequenced and aligned against the transcript and genomic sequences of chickpea. The 100% correspondence of the sequence between the amplicons and those of the reference transcriptome and genomic sequences of chickpea confirmed the on-target amplification of the chickpea homolog. We designated this gene as CaStGR1 (for *Cicer arietinum Stay-Green* gene 1)

2.5.1.3. Association of CaStGR1 Sequence Variants with Green Cotyledon Chickpea Germplasm

Examination of the nucleotide sequence of the green-cotyledon line PI 450,727 indicated a single nucleotide (1-bp) deletion within the first exon of CaStGR1. This frameshifting mutation is predicted to result in missense changes (from amino acid residue 34) coupled with premature termination of translation (at amino acid residue 56) of 266 amino acid residues of a full-length, functional 'wild type' CaStGR1 protein.

To determine the prevalence of delayed degreening and of nucleotide variation in CaStGR1 more broadly among chickpea germplasm, we examined the rate of degreening in a set of 53 chickpea lines in total (Supplementary Table S1). This collection was predominantly germplasm from the US gene bank (34 accessions) that was supplemented with breeding lines (15 genotypes) and cultivars with green cotyledon color, with a smaller number of normal, tan/yellow cotyledon lines serving as controls (Supplementary Table S1).

A total of 33 genotypes of which 27 possessed green cotyledons, including genotypes PI 450,727 and W6 25,975 which were analyzed previously, along with six additional genotypes with yellow cotyledons, were assessed phenotypically in a leaf

degreening assay. In this analysis, all of the 27 green cotyledon genotypes exhibited delayed degreening, whereas by contrast, all six of the yellow cotyledonary lines senesced rapidly with yellowing of detached leaves by day five of the experiment. Furthermore, the degreening phenotype of the 27 with green cotyledons were indistinguishable from that of the previously characterized genotypes PI 450,727 and W6 25,975 that were included alongside in this analysis. This invariant association between green cotyledon color and delay in degreening of detached leaves suggested that the additional 25 germplasm lines may harbor similar molecular variation previously observed in genotypes PI 450,727 and W6 25975.

PCR amplification with CaStGR1-specific oligos with genomic DNA as the template was conducted in 41 genotypes, of which 37 were green cotyledonary with the remaining four with yellow cotyledons. Amplification was consistently unsuccessful in 10 green cotyledon genotypes despite exhaustive PCR attempts, in a manner similar to that in the presumptive large-deletion in genotype W6 25,975 (Supplementary Table S1). Sanger sequencing of PCR amplicons revealed the presence of the 1-bp deletion previously identified in genotype PI 450,727 in an additional six genotypes (Supplementary Table S1 and Supplementary Figure S1). We designated this variant as CaStGR1-1 allele. Of the remaining 25 genotypes, the four genotypes with yellow cotyledons each had a nucleotide identical to that of 'wild type' staygreen gene (that we designated as allele CaStGR1), whereas the remaining 21 genotypes with green cotyledons contained either one of three nucleotide variants in the coding region of the CaStGR1 gene (Supplementary Table S1). Accession ICC 16,340 that was used as the source for breeding of green cotyledon chickpea at ICRISAT-India, along with four breeding lines (also from the ICRISAT-India chickpea

breeding program) all shared a novel 8-bp deletion in exon 2 (Supplementary Table S1 and Figure S1) that we designated as allele CaStGR1-2. Ten other genotypes (9 germplasm accessions and the Canadian green-cotyledon cultivar "CDC Verano") shared another molecular lesion, consisting of a 1-bp deletion (Supplementary Table S1 and Supplementary Figure S1) that we designated as allele CaStGR1-3. Although this variant is also located within exon 2 of CaStGR1, it falls downstream in the coding sequence of the location of the 8-bp deletions observed among material from ICRISAT (allele CaStGR1-2; Supplementary Figure S1). The remaining six green cotyledon genotypes, that included three germplasm accessions and three breeding lines from the USDA-ARS breeding program in Pullman, Washington, USA, each harbored yet another molecular variant, in the form of a 1-bp deletion in exon 4 of CaStGR1 (Supplementary Table S1 and Supplementary Figure S1) which we designated as allele CaStGR1-4). Taken together, the PCR amplification and amplicon sequencing data identified five different molecular lesions in CaStGR1 (Figure 1a and Supplementary Figure S1) that occur exclusively among green cotyledon genotypes (Table 1 and Supplementary Table S1).

2.1.4. Whole Genome Skim Sequencing Delimits the Extent of the Deletion in Allele CaStGR1-5

The absence of amplification in genotypes with the CaStGR1-5 allele with oligonucleotide primers located within the entire coding regions of CaStGR1 was suggestive of a larger sized deletion. To characterize the extend of this deletion we focused on genotype W6 25,975 that typifies this large-deletion allele. In an initial experiment, using the draft whole genome of chickpea genotype CDC-Frontier [50] as a guide, oligos sited in low copy sequences immediately adjacent (within few kbp) to CaStGR1 were

designed and used in PCR amplification. Amplification products of the expected size (3-6 kbp in length) were consistently obtained from wild type ICCV 96,029 genotype and PI 450,727 harboring a 1-bp in exon 1 (allele CaStGR1-1). By contrast, no amplification products were obtained from W6 25975, indicating a deletion of larger and yet to be determined size.

To further characterize the extent of this deletion, a whole genome shotgun library was prepared using genomic DNA of the green cotyledon genotype W6 25,975 and sequenced with Illumina HiSeq platform. Sequences obtained were aligned against short read data from normal yellow cotyledon genotypes ICCV2, ICC 16,207 and ICCV 96029, and anchored to the draft whole genome sequence of chickpea genotype CDC-Frontier [50]. Analysis of the resulting pileup of short-read data localized the wild type CaStGR1 gene to between positions 2.047 and 2.049 Mbp on chickpea chromosome 8′s pseudomolecule (Figure 2b). This multi-genotype sequence pileup data suggested a deletion of ~25 kbp in length, from ~2.026 Mbp within an adjacent predicted gene on one side, through CaStGR1 at ~2.047 Mbp, and into another predicted gene at ~2.052 Mbp on the other side of CaStGR1 (Figure 2 b). Oligonucleotide primers were designed in the low copy predicted genes at ~2.026 Mbp and ~2.052 Mbp that flank CaStGR1, to encompass the ~25 kbp deduced deletion. PCR amplification with these deletion-spanning oligos yielded amplification products of the expected size (3–6 kbp) in genotype W6 25,975 but not in PI 450,727 (where the amplicon would be >25 kbp in size, beyond the capacity of PCR conditions used). The whole genome skim sequencing data together with the PCR results with the gap-spanning oligos corroborate that the CaStGR1-5 allele represents a

large deletion of ~26 kbp in size that encompasses the entirety of the CaStGR1 gene (Figure 2b).

2.1.5. Genetic Cosegregation of Staygreen Sequence Variants with the Green-Cotyledon Trait

In two F2 populations that we examined, the green cotyledon trait segregates as a monogenic recessive trait. In the PI 450,727 × RS11 F2 population, of 47 F2s 35 were of yellow cotyledon color with the remaining 12 with green cotyledon color. In a second F2 population of 88 individuals derived from a cross between yellow cotyledon cultivar 'Royal' and the green cotyledon accession PI 359555, 63 F2s had yellow cotyledons and the remaining 25 F2s had green colors. These fit the 3:1 ratio that is expected for a monogenic recessive gene in the F2 generation (with chi-square values of 0.007 and 0.545; and *p*-values 0.933 and 0.460 for the PI 450,727 × RS11 and Royal × PI 359,555 F2 populations respectively).

The single nucleotide deletion identified in the green cotyledon accession PI 450,727 creates a Hpy-188I restriction enzyme recognition site, which allowed for the design of a CAPS (cleaved amplified polymorphic sequence) marker for the CaStGR1-1 variant allele. A F2 population of 47 individuals, derived from a cross between PI 450,727 (with green cotyledons) and accession RS11 (with normal yellow cotyledons), was phenotyped for cotyledon color and genotyped with the Hpy-188I CAPS marker. In this analysis, all 12 F2 individuals with green cotyledons were homozygous for the PI 450,727 allele, while the remaining 35 F2 individuals were either heterozygous or homozygous for

the yellow cotyledon allele of RS11, as would be expected for a monogenic recessively inherited trait conditioning green cotyledon color.

We further examined cosegregation between cotyledon color and molecular variation in the CaStGR1 gene in additional F2 populations. A green cotyledonary breeding line with the CaStGR1-4 allele was crossed to the elite cultivars 'Nash' and 'Billybean' from which F2 populations were generated. Seeds of these F2s were scored for cotyledon color prior to sowing, and subsequently degreening of vegetative leaves assessed by the foil wrap assay. A KASP marker assay for the 1-bp deletion that occurs in this allele was developed and used to genotype these F2 individuals, and to examine the correlation with the seed cotyledon color and degreening phenotypes. In this analysis, all 52 individuals with green cotyledons and delayed degreening of leaves were homozygous for the 1-bp deletion allele. Of an additional 55 individuals with yellow cotyledons and rapid degreening of leaves, 24 individuals were homozygous for the wild type allele, with the remaining 31 individuals heterozygous for the two alleles. These observations are consistent with the expected monogenic recessive nature expected for the CaStGR1-4 allele. The loss-of-function of the protein in the 52 homozygotes for the deletion allele engendering phenotypes on seed color. By contrast, the presence of one or more of the wild type alleles in the other 55 individuals provides a functional protein, and the associated normal yellow cotyledon color and normal rate of degreening.

2.5.2 Characterization of Physiological Functions of Green Cotyledon Chickpea

The genetic and early phenotypic analysis indicated that green cotyledon chickpea is sharing a common suite of characteristics such as delayed degreening in leaf tissue, and

which were in contrast to those observed in regular yellow cotyledon chickpeas. To determine the impacts of altered function of the chickpea stay-green gene in these green cotyledon lines, we undertook a set of studies to characterize the impacts on plant physiological functions and indicators of agronomic performance.

2.5.2.1. Plant Responsiveness to Soil and Atmospheric Drought (Experiment 1 and 2)

The main purpose of the response to soil and atmospheric drought experiments (experiment 1 and 2) was to characterize the crop capacity to restrict transpiration upon severing soil/atmospheric moisture deficit. The plant responsiveness to soil moisture deficit could be expressed as the soil moisture threshold (i.e., fraction of transpirable soil water; FTSW) when the plant transpiration significantly declines compared to transpiration of WW plants. Across the experiments, we documented a wide range of the genotypic responses to declining soil moisture. FTSW values of 0.43–0.64 were observed among germplasm (Figure 3a), which encompassed the narrower range of FTSW (0.54-0.58) observed in stay-green introgression lines (ILs) that originated from the Indian elite cultivars KAK2 and JKG1 (Figure 3b and Table 2). Within the germplasm lines, genotypes with functional StGR1-WT allele tended to limit their transpiration at a higher level of soil moisture (FTSW threshold higher than 0.5) although we couldn't statistically differentiate these lines from the other tested StGR1 allelic variants. In the series of experiments with introgression lines (ILs) based on Indian elite cultivars (KAK2 and JKG1), we found that FTSW thresholds of both cultivated recurrent parents (KAK2 and JKG1) was very narrow (0.54 ± 0.03) and significantly lower compared to the FTSW of the stay-green trait donor parent ICC 16,340 (0.58 ± 0.02) whereas there was no significant difference between ILs and the parental lines.

Further, we tested the plant's capacity to regulate transpiration rate (TR [g of water transpired per cm-1 of canopy per h]) in conditions of a drying atmosphere (i.e., increasing vapor pressure deficit; VPD). Here, we documented wide range of variability in the tested material and across the range of conditions (outdoors typically ~0.5–3.0 kPa [Figure 4a and 4b] and in growth chambers 1.2 to 4.6 kPa [Figure 5a and 5b]). TR responses to VPD under natural atmospheric (outdoor) conditions and under controlled VPD (growth chamber) conditions showed a similar trend (Figures 4a,b and 5a,b; Table 3). In germplasm, we found no consistent trend in material with ("wild type") stay-green allele or without (i.e., Loss-of-Function alleles CaStGR1-1 to CaStGR1-5) in the TR responsiveness to VPD (Figure 5a). Some StGR1 loss-of-function germplasm allelic variants were having TR higher while others lower than values observed for germplasm with a functional (wild type) stay-green gene. In experiment 2b and 2c's series encompassing the stay-green ILs, we found the stay-green donor ICC 16,340 had a higher TR and rapid TR increase upon increasing VPD compared to the recurrent elite parents and their stay-green derivatives in both outdoor and controlled (growth chamber) conditions (Figures 4a, 4b, 5b; and Table 3). Interestingly, whereas ILs with the KAK2 background had TR and VPD values intermediate to those of the stay-green donor line ICC 16,340 and the elite cultivar KAK2 (Figure 4a), all the stay-green derivatives of JGK1 had even significantly lower TR across the VPD regimes compared to JGK1 elite parent (Figure 4b). Furthermore, in well watered (WW) conditions, there were no significant genotypic differences in the specific leaf weight (SLW) in germplasm, the JKG1-derived ILs had lower SLW compared to both of the parents (Supplementary Figures S2a,b).

2.5.2.2. Variation in Plant Growth and Water-Use Related Traits in Lysimetric Facility (Experiment 3a, b)

In the lysimeter experiment under well-watered (WW) conditions with germplasm (Experiment 3a) and introgression lines (Experiment 3b), significant genotypic differences in the total amount of water required to reach maturity were observed (data no shown). However this was mostly conditioned by the different phenological development between germplasm and the ILs. The relationship between total water use and days to flowering was strongly correlated in germplasm ($R^2 = 0.63$*; Supplementary Figure S3a) but only very weakly in the introgression lines ($R^2 = 0.10$ns; Supplementary Figure S3b). Under water stress (WS) differences in total amount of water extracted from lysimeters was independent of crop phenology but these did not coincide with the presence of particular CaStGR1 allele in any of the material used.

Under WW and WS, although differences were observed in total biomass accumulation and seed setting, these did not appear to be associated with the stay-green trait. However, the relative decline in total biomass accumulation due to water stress was very similar between all allelic variants with reduction in WS when compared to WW, of ~50% in germplasm and ~30% in IL material. In experiment 3b under WW treatments, the seed yield was largely explained by duration of phenological stages (Supplementary Figure S4). Interestingly in the same experiments under WS, the seed yield did not relate to crops phenology (Supplementary Figure S4) but related positively to seed number ($R^2 = 0.66$* in ILs; experiment 3a, $R^2 = 0.73$ in germplasm materials). In addition, TE [g biomass per kg of water transpired] and seed yield were strongly associated under WS conditions [$R^2 = 0.62$ *** in ILs (Figure 6a) and $R^2 = 0.37$ in germplasm], while there was a weak

relationship between TE and seed yield under WW conditions (Figure 6b). Also, in experiment 3b under WS, there were several stay-green isolines in each elite genetic background, which had seed yield comparable or higher than the respective elite recurrents and stay-green donor (Supplementary Figure S5).

2.5.2.3. Evaluation of Canopy Growth Related Traits (Experiment 4)

The canopy growth parameters were examined only among stay-green ILs alongside the donor germplasm line ICC 16,340 and the recurrent elite cultivars JGK1 and KAK2. We found the donor parent ICC 16,340 had lower canopy growth rates than elite recurrent parents (JGK1, KAK2) with some of the ILs attaining higher growth rates compared to stay-green donor parent and recurrent parents (Figure 7a) and this reflected in the differences in canopy size and digital biomass estimates averaged across the time of observations (Figure 7b). The parental line JGK1 grew more slowly compared to the elite recurrent line KAK2 (Figure 7a). The stay-green derivative ILs in the KAK2 elite cultivar background had growth rates similar to those of the recurrent elite parent KAK2 (Figure 7a). Growth rates in stay-green ILs originated from the elite cultivar, JGK1 exceeded those observed in both parents, and at levels similar to those of in KAK2 stay-green ILs. This indicated that stay-green IL material had recovered its vigor (Figure 7a).

2.5.2.4. Evaluation in the Field Conditions (Experiment 5)

The IL plant material that was relatively more homogeneous for the main phenology-related characters was tested in the field alongside their recurrent parents (experiment 5; flowering 37–53 DAS, days to maturity 97-101). Some of the tested ILs attained similar or even higher grain yield under irrigated conditions (Figure 8a), which

was partially positively driven by phenology differences [Relationship between accumulated biomass or seed yield and days to flowering; $R^2 = 0.51$* (Supplementary Figure S6a) and negatively related to harvest index [Relationship between accumulated biomass and harvest index (HI); $R^2 = 0.30$* (Supplementary Figure S6b)]. Water stress (WS) conditions reduced the grain yield cca 40–70%. Under WS conditions, the yield of stay-green ILs in relation with the days to flowering was much looser (Supplementary Figure S6c). We also observed the lack of correlation between the production traits (biomass and yield) and phenology parameters [Regression between accumulated biomass and days to flowering; $R^2 = 0.0001$ & regression between seed yield and days to flowering; $R^2 = 0.08$] while the relation between HI was maintained [Relationship between seed yield and harvest index (HI); $R^2 = 0.21$ (Supplementary Figure S6d)]. Interestingly, we found that the extent of yield reduction due to WS was similar between the parental lines and some of the stay-green introgression line progenies (Figure 8b), and was further positively related to plant capacity to grow in WW but negatively in WS (i.e., higher production potential, higher yield reduction due to WS while the "smaller" plants had suffered less yield reduction under WS).

2.5.2.5. Leaf Pigments Content Under WW and WS Conditions (Experiments 1c, 3a,b,c)

Pigments in the Leaf Tissues and Grains.

Across all material tested, we found that plants grown outdoors (in lysimeters, experiment 3c) maintained much higher levels of photosynthetic pigments, especially carotenoids in leaves tissues, compared to plants cultivated in the glass-house (in lysimeters, experiment 3b) environments .

We found no differences between the levels of leaf pigments (i.e., chlorophyll a, chlorophyll b, total carotenoids) and their ratio (chlorophyll a/chlorophyll b ratio) in the materials carrying the CaStGR1-wt functioning allele and CaStGR1-1 to 5 malfunctioning allele (ILs and some germplasm) under WW. The methodology of stress imposition and the tissue sampling (the last fully developed leaf on the main stem) couldn't discriminate the stay-green material from wild-type under the WS conditions either. However, we found a higher chlorophyll_a and cholorophyll_b content in mature seeds of material carrying stay-green alleles compared to CaStGR1-wt in both germplasm (Supplementary Figure S7a,b) and stay-green ILs (Supplementary Figure S8a,b). Similarly, the grain total carotenoids content was ~10–30% higher in the stay-green loss-of-function variants (alleles CaStGR1-1 to 5) compared to wild type (CaStGR1-WT; Figure 9a) in germplasm and ILs. Furthermore, grain total caratenoid levels were not significantly affected by the conditions of cultivation (WW and WS) in the introgression lines (Figure 9b).

The detailed fractionation of carotenoids contents in ILs seeds revealed that there were ~3-fold higher levels of lutein and beta-carotene (provitamin A) in the seeds of green cotyledon introgression lines (ILs) compared to both of the yellow cotyledon colored elite cultivars (KAK2 and JGK1; Figure 10). By contrast, zeaxanthin content did not significantly vary between ILs with green cotyledons and the elite cultivars with yellow cotyledons (KAK2 and JGK1; Figure 10) used as recurrent parents in introgression line development.

2.6 Discussion

The two goals of the present study were to (1) understand the molecular and functional mechanisms underlying the delayed senescence in chickpea with the "cosmetic stay-green" trait [29,31] and, (2) to characterize the effects of the "cosmetic stay-green trait" on plant performance in semiarid agricultural systems. Since the majority of chickpea production occurs under water-limited rainfed conditions, (i.e., terminal drought), understanding responses to water limitations is critical to evaluating the potential of stay-green chickpea. Lastly, we also investigated the nutrient composition of stay-green chickpea, as a genetic biofortification technology to alleviate nutritional deficiencies for carotenoids in consumers.

2.6.1. Identification of 'Cosmetic Stay-Green' Allele in Chickpea

Recent developments in genome sequencing have provided deep sequence resources for several legumes, in terms of whole genome sequences and transcriptomes. These sequence data provide a valuable resource for both the comparative and evolutionary studies of genome structure and genes. Subsequent analysis of amplified chickpea sequences and their localization to the chickpea draft genome supported the identification of the cognate chickpea stay-green gene that exhibited a high degree of sequence similarity with the other legume stay-green orthologs, and localized to a syntenic position on chromosome 8 in the chickpea draft genome [50]. This genomic region corresponds to the large-effect QTL for carotenoid concentrations described among three F2 populations of chickpea [39], which contains the staygreen gene ortholog (LOC101509366; [39]). Our methods highlight the utility of draft or reference genomes for the more detailed study of

individual genes from their initial identification to deduction of orthology from the evolutionary history.

In addition, we also conducted a whole genome skim sequencing, to delimit the extent of the deletion in allele StGR1-5. Initial and exhaustive PCR amplifications indicated this allele as probably encompassing the entire coding region of the chickpea ortholog, but whose boundaries were unknown. The use of whole genome skim sequencing of the genome for this allele allowed us to flank the large (several 10s of kbp; Figure 2b) deletion in a single experiment. This contrasts with earlier approaches such as primer amplicon 'walking' that given the large size of the deletion would not have yielded results or required the use of a large collection of oligos at varying distance surrounding the StGR1 gene.

The monogenic recessive nature of the green cotyledon trait is supported by observation of only yellow cotyledon phenotypes in the F1 individuals from crosses between yellow and green cotyledon chickpeas, and in cosegregation data in segregating progenies (described in results). Furthermore, the occurrence of green cotyledon phenotype in F1s obtained from crosses among alleles, and invariantly green cotyledon in their F2s supports our inference that the five molecular variants we identified and describe in this study comprise an allelic series in StGR1 gene.

The recessive behavior of the green cotyledon alleles of chickpea is consistent with a loss-of-function of the chickpea StGR1 gene in these phenotypic variants. This inference is corroborated by the likely impact of the deletions on the deduced amino acid sequence of the translated protein. The single nucleotide deletions in alleles StGR1-1 to allele

StGR1-4 all occur within the coding regions, and consequently these deletions would result in a frame shift of the open reading frame (and premature truncation of the translated protein).

The identification of five different loss-of-function alleles in CaStGR1, and the absence of nesting (where more than one deletion allele occurs within a single genotype), implies that the green cotyledon trait arose independently at least four times in chickpea, and as naturally occurring variation among chickpea germplasm. The fifth gene-encompassing deletion allele StGR1-5 could represent a fifth independent origin of green cotyledon trait in chickpea. However, based on our data we cannot preclude the possibility that this allele may have arisen secondarily within the background of one of the other small 1-bp deletion alleles (StGR1-1 to StGR1-4). Additional analyses of the green cotyledon germplasm along with related germplasm might help to clarify this current ambiguity.

It is intriguing that green cotyledon breeding lines from the three different chickpea breeding programs (ICRISAT in India, USDA-ARS in USA, and the University of Saskatchewan in Canada) represent three different and distinct loss-of-function alleles of the stay-green gene as a source of the green cotyledon trait. This could be a reflection of the limited knowledge or availability of the sources of green cotyledon germplasm in these breeding programs. Alternatively, the use of the different alleles in each breeding program might reflect preferential use of distinct germplasm on the basis of other traits (e.g., for local adaptation, market type, disease reactions) present in the various germplasm sources. Indeed, our observation of varying phenology among green cotyledon germplasm could represent such additional phenotypic variation, along with seed size and color that also

vary. In such a scenario, the distinct alleles for StGR1 gene are merely inadvertently co-selected for a desired common trait of green cotyledons from germplasm with additional characteristics.

Despite the recurrent selection at an orthologous StGR gene in multiple crop legumes for green cotyledon color, it is possible that additional genes exist that replicate this phenotype, or might modulate it. For example, in the more exhaustively studied Rice and *Arabidopsis* systems (e.g., [29,51–53]), genes other than the stay-green protein have also been implicated in the cotyledon color or persistence of chlorophyll machinery which would affect stay-green phenotypes. Furthermore, some aspects of the green cotyledon trait, and its manifestation at the level of whole seeds is also likely to depend on pigmentation in the overlying seed coat tissues. For example, in cowpea, distinct genes controlling green color in cotyledon and green color in seed coats have been described [42,54].

Our identification of the molecular nature of variation among green cotyledon chickpea should facilitate the use of molecular marker assisted selection (MAS) or backcrossing (MABC) for introgression of this trait in chickpea breeding. For example, in the current study we developed and tested a KASP marker for the StGR1-4 allele found in USDA-ARS breeding lines (Supplementary Tables S1 and S2). This assay is effective at monitoring the allele states (wt or 1-bp deletion) within exon 4 of the chickpea gene, and is being used for marker-assisted backcrossing in our program. Design and testing of similar KASP assays for the remaining single nucleotide deletions (alleles StGR1 -1, -2, -3) is being planned to facilitate similar use of MAS with these distinct allelic variants.

2.6.2. Green Cotyledon Trait as a Vavilovian Homologous Series of Variation

Green cotyledon market classes or types occur in several crop legumes, including garden pea [52], Medicago [33], chickpea [30,55], common bean [52], lima bean [52], and cowpea [54]. This recurrence suggests that the green cotyledon color trait arose from the repeated and independent selection from the white or yellow cotyledon forms that typify these crops and their wild relatives. The prevalence of repeated human selection for a common phenotype in multiple crops was suggested by the pioneering crop evolutionary botanist Nikolai Vavilov [56].

2.6.3. Stay-Green Alleles do not Affect the Plant Responsiveness to Soil and Atmospheric Drought

2.6.3.1. Plant Responsiveness to Soil and Atmospheric Drought

Any novel crop technology intended for practical utilization in complex agrisystems has to be appropriately tested to enhance the probability to be implemented and accepted. In many of the semiarid rain-fed agrisystems, one of the main limiting factors to crop productivity is soil moisture deficit [2,8,57–60]. To understand plant responses to decreased soil moisture, we have generated substantial evidence on plant functions that contribute to crop adaptations in these environments [61–65]. In the present study we evaluated whether stay-green phenotype in chickpea underlined by CaStGR1 gene might be functionally involved in any important environmental adaptations (i.e., responsiveness to soil and atmospheric drought). We found that in all tested material carrying the stay-green CaStGR1 gene (germplasm or cultivated crop types) we did not observe any association between allelic variation and plant responsiveness to soil/atmospheric drought

which would have impacted crop production in dry environments. In the cultivated plant types, we found that CaStGR1-2 stay-green ILs inherited the level of environmental adaptations from the cultivated parent rather than from the donor of this stay-green allele (ICC 16340). In some particular cases, the level of adaptive features was even more pronounced than in the cultivated recurrent parent (JGK-1 and derived ILs; Figure 4b). We speculate that this "transgressive segregation" could have been, at least partially, driven by the higher capacity to grow and expand canopy of ILs originated from this cross (Figure 7b; see [65]).

2.6.3.2. Plant Water-Use Related Traits and Agronomic Performance

Crop functions linked to quantity and efficiency of water utilization (e.g., see above) determines its agronomic performance, especially in environments limited by the water availability [10,66,67]. As discussed above we showed that CaStGR1 allelic variation does not appear to affect the relatively simple plant functions which were previously documented to influence crop adaptations to dry environments [2,59,68]. However, since crop yield is a very complex trait, we have also tested the CaStGR1 allelic variants in the systems relevant for evaluation of crop agronomic characteristics (i.e., lysimteric system and field).

We found there were significant differences in grain and biomass yield in germplasm when tested under different irrigation regimes but none of the differences seemed to coincide with the presence of disrupted CaStGR1 allele (CaStGR1-1 to CaStGR1-5). These differences in germplasm production characteristics were mostly explained by the differences in phenological development. In the stay-green CaStGR-1-2

ILs derived on cultivated background, we found significant genotypic differences in the main production parameters with the recurrent parents attaining generally higher production (example on Figure 8a). Nevertheless, in each of these experiments there were at least few ILs in the genetic background of each of the two elite cultivars whose production was comparable to the elite recurrent parents under WW and WS treatment (which ILs were consistent). Interestingly, under WS treatment, yield of some ILs was similar to that of their respective recurrent parents despite the phenological development of these ILs was generally several days longer (~14 days). Further, we found that the relation between seed yield and flowering time was much looser than that of the germplasm (as in [69,70])-especially under WS where this correlation was hardly significant (e.g., Supplementary Figure S6). However, we found that the majority of variation in grain yield and yield components within this material was explained by TE, especially under WS (Figure 6a, b). We can speculate that higher TE in some of the tested ILs could have been the consequence of lower TR and increased transpiration responsiveness of some ILs to VPD (see above and Figure 4b). We can further speculate that the enhanced TE of some tested ILs could be a consequence of yet to be determined mechanisms induced by portions or interactions of genome remaining from the donor genotype since the recurrent background of IL material was not completely recovered at BC4-5:F2 (i.e., ~94–97% of recurrent background recovered).

Collectively these data indicate that across the range of tested conditions there is no significant trade-off between elevated carotenoid content and agronomic productivity. Yields were similar between lines with “wild type” CaStGR1 (with yellow cotyledons) and

genotypes with loss-of-function alleles in the CaStGR1 gene (with green cotyledon and delayed degreening phenotypes).

2.6.4. Stay-Green Alleles Extend Retention of Chlorophyll and Provitaminogenic Carotenoids in Grains and Leaves

Several stay-green plant phenotypes have been described in different crops [52]. The common denominator of "stay-green" phenotype can be described as a plant's capacity to remain green (i.e., maintain chlorophylls) in particular circumstances (reviewed [29,71,72]). In general, we can consider two basic stay-green types; "cosmetic" and "functional". Cosmetic stay-green is underlined by any mechanism that avoids chlorophylls to degrade—therefore the plant tissues appear green even if desiccated. Functional stay-green is a consequence of plants ability to manage resources during the crop cycle (e.g., water and nitrogen; [8,25,27,28,73,74]).

We present evidence that the green-seeded chickpea material is of a "cosmetic" type and depended on the presence of disrupted CaStGR1 gene, an ortholog of Mendel's I locus of garden pea (see above), that affects the function of chlorophyll degrading enzyme [48] and resulted in retention of chlorophylls in dried plant tissues (grain and leaf). We were further interested in addressing whether the composition of chlorophylls *a* and *b* and the functionally related pigments (carotenoids) differed among plant tissues (grain and leaves) during a range of circumstances (irrigated and water stress).

Consistently, we found that the levels and the composition of pigments did not significantly differ between genotypes carrying disrupted CaStGR1 gene (allele 1–5) and wild-type under irrigated and even under water stress conditions (probably because for this

estimation only the leaves from the top of the plants which still remained green even in wild-type were sampled). Nevertheless, we found that all stay-green genotypes, in general, maintained higher level of pigments in matured grains compared to wild-type in irrigated conditions (similarly in [30]). The pigments in the grain were not significantly affected by the conditions of cultivation (WW and WS) across the range of material tested and the grains produced by plants exhibiting stay-green phenotype had all 10-100% higher chlorophyll and total carotenoids contents compared to the respective wild-type checks (similarly in [30,75]). Further dissection indicated the stay-green ILs contained two to three fold higher levels of specific A-provitaminogenic carotenoids (beta-carotene) resembling or exceeding the levels achieved by "golden-rice" technology [39,76].

Additional studies are required to determine the extent to which these elevated levels of carotenoids translate into enhanced bioavailability of vitamin A for humans, factors influencing consumer acceptance of green cotyledon colored chickpeas as dry grains, and if green cotyledon chickpea may be associated with conditionally-reduced seed germination or seedling establishment as has been observed in some other crop legumes.

2.6.5. Conclusions

Chickpea production suffers greatly due to its cultivation predominantly as a rain-fed crop, particularly across developing countries. Significant progress has been made from crop agronomic practices and breeding to address the yield gap to ensure appropriate caloric intake of populations inhabiting these areas. Although caloric intake is slowly increasing, human nutrient deficiencies prevail in the same regions and remain largely unaddressed. Therefore, in this paper we tested the suitability of stay-green chickpea for

cultivation in semiarid tropical regions, which as a genetic biofortification technology may help to reduce widespread vitamin-A deficiency while maintaining the levels of agronomic production. We tested a range of plant material with the stay-green character which was expressed as an extended maintenance of chlorophylls and carotenoids in dry seeds and leaves. We found this particular phenotype was controlled by variation in a single gene, CaStGR1, an ortholog of Mendel's I locus of garden pea, which occurred in 5 different allelic variants in the tested material. We also showed that across a range of environmental conditions the stay-green allelic variants were very likely neither influencing the mechanisms linked to drought stress adaptations nor negatively influencing important agronomic traits. Our evidence that the green-seeded CaStGR1 variants contain multiple-fold higher levels of the phytonutrients lutein, and provitamin A (beta-carotene) when compared to the more common yellow cotyledon chickpea indicate a higher nutritional value of the green cotyledon type. Further investigations of the bioavailability of vitamin A, multilocation trials for yield stability, and acceptability of the stay-green chickpea products in production regions by producers and consumers are warranted in order to establish the efficacy of genetic biofortification with stay-green chickpea for improving human nutrition and health.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/20/22/ 5562/s1.

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2.8 Tables

Table 2.1 Summary of nucleotide variants identified in CaStGR1 among chickpea germplasm. The color of cotyledons, designated allele names for the variants, the nature of molecular lesions found in each allele, and their frequencies among germplasm studied are listed

Cotyledon Color	Allele	Nucleotide Variation in CaStGR1	Number of Genotypes
green	CaStGR1-1	1-bp "g" del in exon 1	7
green	CaStGR1-2	8-bp "ctaggttg" deletion in exon 2	5
green	CaStGR1-3	1-bp "c" deletion in exon 2	10
green	CaStGR1-4	1-bp "g" del in exon 4	6
green	CaStGR1-5	entire gene deleted	11
Yellow/Tan	CaStGR1 WT	"Wild Type"	6

Table 2.2 Regression analysis of transpiration response to soil drying of green cotyledon trait donor genotype ICC 16340, recurrent yellow cotyledon elite cultivars KAK-2 and JGK1 and backcross introgression lines of the green cotyledon trait in these elite cultivar backgrounds.

Genotypes	NTR-FTSW Thresholds and Std. Error	Slope 1 and Std. Error	Slope 2 and Std. Error
ICC 16340_Stg-D-P	0.58 ± 0.02	1.92 ± 0.08	−0.59 ± 0.23
KAK2_R-P	0.54 ± 0.03	1.72 ± 0.05	0.20 ± 0.26
JGK 1_R-P	0.54 ± 0.03	1.88 ± 0.06	−0.12 ± 0.24
ICCX-060119-107 (KAK2)	0.54 ± 0.03	1.85 ± 0.08	−0.10 ± 0.22
ICCX-060119-113 (KAK2)	0.48 ± 0.03	1.98 ± 0.09	0.06 ± 0.17
ICCX-060119-116 (KAK2)	0.58 ± 0.03	1.64 ± 0.07	0.01 ± 0.19
ICCX-060119-123 (KAK2)	0.62 ± 0.05	1.69 ± 0.09	−0.32 ± 0.53
ICCX-060121-125 (JGK1)	0.51 ± 0.02	1.87 ± 0.06	−0.01 ± 0.17
ICCX-060121-128 (JGK1)	0.60 ± 0.03	1.63 ± 0.06	0.05 ± 0.28
ICCX-060121-129 (JGK1)	0.55 ± 0.03	1.72 ± 0.07	0.14 ± 0.18

Table 2.3 Regression analysis of transpiration response to VPD in outdoor and growth chamber of green cotyledon trait donor genotype ICC 16340, recurrent yellow cotyledon elite cultivars KAK-2 and JGK1 and backcross introgression lines of the green cotyledon trait in these elite cultivar backgrounds.

Genotypes	TR Response to VPD at Outdoor		TR Response to VPD at Growth Chamber		
KAK-2 Background	**Mean TR & SE LSD (0.01) = 0.09**	**Slope at high VPD & SE LSD (0.01) = 0.59**	**Mean TR & SE LSD (0.001) = 0.35**	**Slope & SE LSD (0.01) = 0.04**	**R^2**
ICC 16340_Stay-green_Donor Parent	1.31 ± 0.04a	5.48 ± 0.18a	2.02 ± 0.06a	0.52 ± 0.06a	0.94
KAK2_Recurrent parent	0.95 ± 0.02b	4.18 ± 0.08b	1.45 ± 0.06b	0.41 ± 0.04b	0.94
ICCX-060119-107 (KAK2 Background)	0.98 ± 0.04b	4.16 ± 0.18b	1.37 ± 0.04b	0.43 ± 0.04b	0.95
ICCX-060119-113 (KAK2 Background)	1.04 ± 0.02b	4.23 ± 0.06b	1.49 ± 0.06b	0.46 ± 0.04b	0.95
ICCX-060119-116 (KAK2 Background)	0.94 ± 0.04b	4.19 ± 0.20b	1.45 ± 0.08b	0.47 ± 0.05b	0.94
ICCX-060119-123 (KAK2 Background)	1.03 ± 0.02b	4.16 ± 0.16b	1.63 ± 0.04b	0.40 ± 0.05b	0.91
JGK-1 Background	**Mean TR & SE LSD (0.001) = 0.12**	**Slope at high VPD & SE LSD (0.01) = 0.46**	**Mean TR & SE LSD (0.01) = 0.25**	**Slope & SE LSD (0.01) = 0.033**	**R^2**
ICC 16340_Stay-green_Donor Parent	1.31 ± 0.04a	5.48 ± 0.18a	2.02 ± 0.06a	0.52 ± 0.06a	0.94
JGK 1_Recurrent parent	1.14 ± 0.04b	4.77 ± 0.16b	1.70 ± 0.06b	0.45 ± 0.04b	0.95
ICCX-060121-125 (JGK1 Background)	0.99 ± 0.03bc	4.09 ± 0.01c	1.53 ± 0.06b	0.42 ± 0.04b	0.95
ICCX-060121-128 (JGK1 Background)	0.90 ± 0.03c	3.82 ± 0.08c	1.52 ± 0.08b	0.38 ± 0.04b	0.94
ICCX-060121-129 (JGK1 Background)	0.90 ± 0.01c	3.78 ± 0.05c	1.52 ± 0.05b	0.37 ± 0.04b	0.94

Supplementary Table S4: Overview of experiments conducted

experimental setup	No of Expt.	Duration of expt.	Plant material	Treatments (WW/WS)	Prevailing weather conditions (VPD;kPa)	Traits measured/derived from the experiment
Pot culture setup for dry-down experiment in glasshouse	Expt. 1a	17-10-2015 to 18-01-2016	Staygreen chickpea germplasm materials (different StGR-alleles)	WW, reps	0.4-2.50 kPa	leaf Senescence
	Expt. 1b	20-01-2016 to 13-02-2016	Staygreen chickpea germplasm materials (different StGR-alleles)	5 replications / genotype / treatment	0.5-2.45 kPa	Daily transpiration, NTR, FTSW and NTR vs. FTSW threshold pint (Break point) and leaf pigments (Chlorophyll_a and Chlorophyll_b, Total carotenoids, Ratio between Chlorophyll a and Chlorphyll b).
	Expt.1c	28-09-2017 to 14-11-2017	Staygreen donor parent and recurrent parent (non-stay-green with high yielding cultuvars) and selected introgression progenies from different background (JGK-1 and KAK-2)	5 WW replications/ genotype 7WS replications / genoptype	0.5-2.65 kPa	Daily transpiration, NTR, FTSW and NTR vs. FTSW threshold pint (Break point) and leaf pigments (Chlorophyll_a and Chlorophyll_b, Total carotenoids, Ratio
Pot culture setup for Transpiration response to VPD in outdoor and growth chamber	Expt. 2a	20-01-2016 to 28-02-2016	Staygreen chickpea germplasm materials (different StGR-alleles)	5 replications / genotype / treatment	1-4.2 kPa	Hourly transpiration, Leaf area, SLW,Transpiration rate, VPD, TR vs.VPD restriction point (Break point), slope 1 and slope 2
	Expt. 2b	28-09-2017 to 27-10-2017	Staygreen donor parent and recurrent parent (non-stay-green with high yielding cultivars) and selected introgression progenies from different background (JGK-1 and KAK-2) are tested in outdoor	8 replications / genotype	0.4-2.85 kPa	Hourly transpiration on outdoor, Leaf area, Transpiration rate and VPD
	Expt. 2c	28-09-2017 to 30-1-2016	Staygreen donor parent and recurrent parent (non-stay-green with high yielding cultivars) and selected introgression progenies from different background (JGK-1 and KAK-2) are tested in growth chamber	8 replications / genotype	1.2-4.6 kPa	Hourly transpiration on growth chamber, Leaf area, SLW, Transpiration rate, VPD, TR vs.VPD restriction point (Break point), slope 1 and slope 2
Lysimetric facility	Expt. 3a	21-11-2015 to 15-02-2016	Staygreen chickpea germplasm materials (different StGR-alleles)	4 replications / genotype / treatment	0.6-2.85 kPa	Weekly transpiration, agronomical traits (TotT, SBM, PWT, PNO,SNO, SW, TE, WUE, DFL_L and DM_L) and grain pigments (Chlorophyll_a and Chlorophyll_b, Total carotenoids, Ratio between Chlorophyll a and Chlorphyll b).
	Expt. 3b	13-12-2016 to 20-03-2017	Staygreen donor parent and recurrent parent (non-stay-green with high yielding cultivars) and selected introgression progenies from different background (JGK-1 and KAK-2) are tested in glass house (indoor)	5 replications / genotype / treatment	0.3-2.9 kPa	Weekly transpiration, agronomical traits (TotT, SBM, PWT, PNO,SNO, SW, TE, WUE, DFL_L and DM_L) and grain pigments (Chlorophyll_a and Chlorophyll_b, Total carotenoids, Ratio between Chlorophyll a and Chlorphyll b).
	Expt. 3c	23-11-2017 to 2-03-2017	Staygreen donor parent and recurrent parent (non-stay-green with high yielding cultivars) and selected introgression progenies from different background (JGK-1 and KAK-2) are tested in outdoor (Rain-out shelter)	4replications / genotype / treatment	0.25-3.2 kPa	Weekly transpiration, agronomical traits (TotT, SBM, PWT, PNO,SNO, SW, TE, WUE, DFL_L and DM_L) and grain pigments (Chlorophyll_a and Chlorophyll_b, Total carotenoids, Ratio between Chlorophyll a and Chlorphyll b).
Leasy-Scan facility	Expt. 4	16-08-2017 to 15-09-2017	Staygreen donor parent and recurrent parent (non-stay-green with high yielding cultivars) and selected introgression progenies from different background (JGK-1 and KAK-2)	4 replications / genotype	0.3-2.8kPa	Canopy development related traits (digital biomass and its growth rate) on daily basis
Field	Expt. 5	16-11-2017 to 28-02-2018	Staygreen donor parent and recurrent parent (non-stay-green with high yielding cultivars) and selected introgression progenies from different background (JGK-1 and KAK-2)	3 replications / genotype / treatment	0.3-4.4 kPa	Phenology & yield and related traits (SBM_F, SY_F, 100_SWT, SNO_F, HI, 50%DFL and DM_F)

Foot notes: NTR-Normalised transpiration ratio; FTSW-Fraction of transpirable soil water; NTR vs. FTSW threshold pint (Break point)-Breakpoints are derived from segmental regression analysis; VPD-Vapour Pressure Deficit, TR vs.VPD restriction point (Break point)-Breakpoints are derived from segmental regression analysis; Slope1 (TR vs.VPD relationship before transpiration restriction) and Slope 2 (TR vs.VPD relationship after transpiration restriction); SE-Stamdard Error; SLW- Specific leaf weight; TotT-Total transpiration; SBM-Shoot biomass; PWT-Pod weight; PNO-Pod numbers; SNO-Seed number; SW-Seed weight; TE-Transpiration efficiency; WUE-Water use efficiency, DFL_L-Days to flowering in Lysimeters; DM_L-Days to maturity in Lysimters; Digital biomass measured by Plant eye camera (Phenospex company); SBM_F-Shoot biomass from field; SY_F-Seed yield from field; 100_SWT-100 seed weight from field, SNO_F-Seed numbers from field, HI-Harvest index, 50%DFL-Days to 50% flowering from field and DM_F-Days to maturity from field).

2.9 Figures

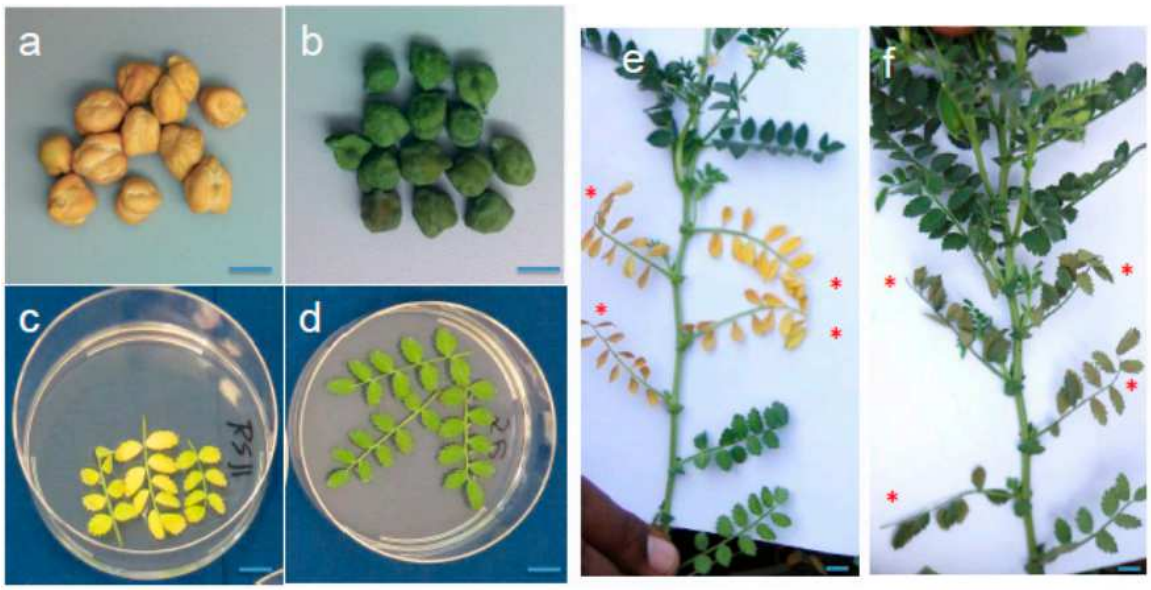

Fig. 2.1 Seed and leaf senescence phenotypes of normal and green chickpea. Dried mature seed of common chickpea with yellow cotyledons (a) and of the green cotyledon colored type (b). Differential degreening rates in detached leaves floated on water after 5 days in the absence of light from normal chickpea (c) and green cotyledon type (d), and from leaves wrapped in aluminum foil from yellow (e) and green chickpea (f). Asterisk in (e) and (f) mark leaves covered by foil for 5 days. Blue lines in each panel corresponds to 1 cm.

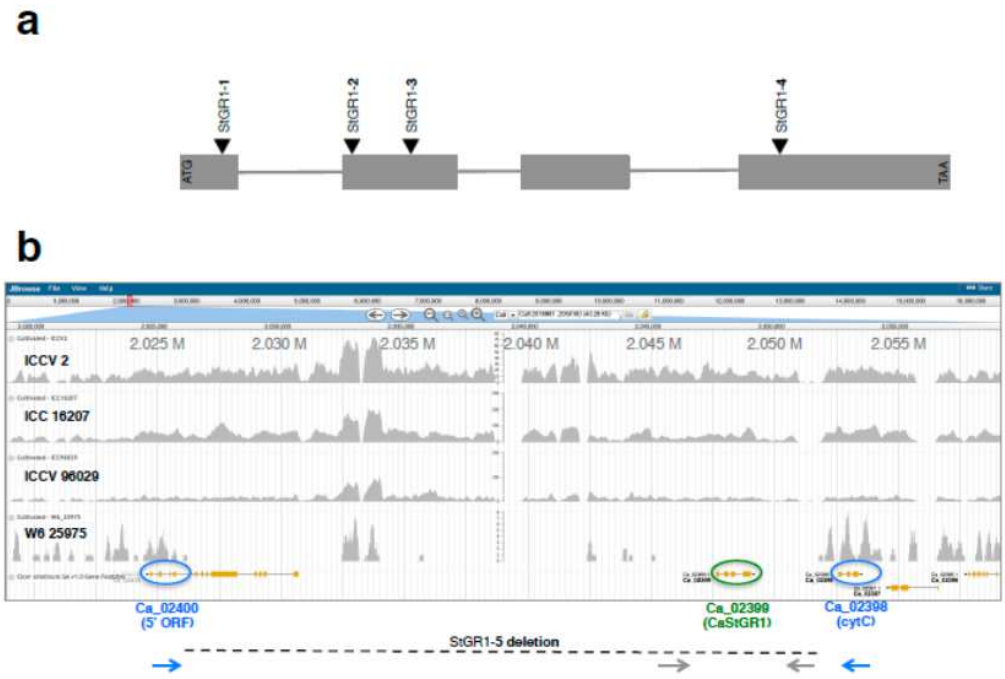

Fig. 2.2 Gene structure and genomic context of type chickpea stay-green gene CaStGR1. (a) Schematic of the gene structure of CaStGR1 are shown in (a), with the four exons denoted by gray boxes and the three introns as thin lines. Locations of the four small deletion alleles CaStGR1 through CaStGR4 are denoted by triangles above the exons. (b) Whole genome Illumina short read skim sequencing read pileups of three normal yellow cotyledon colored chickpea genotypes (ICCV 2, ICC Figure 2. Gene structure and genomic context of type chickpea stay-green gene CaStGR1. (a) Schematic of the gene structure of CaStGR1 are shown in (a), with the four exons denoted by gray boxes and the three introns as thin lines. Locations of the four small deletion alleles CaStGR1 through CaStGR4 are denoted by triangles above the exons. (b) Whole genome Illumina short read skim sequencing read pileups of three normal yellow cotyledon colored chickpea genotypes (ICCV 2, ICC 16,207 and ICCV 96029) are aligned to the chickpea reference of 'CDC Frontier', alongside those from genotype W6 25,975 that harbors the large deletion allele CaStGR1-5. Predicted genes Ca-02399 (CaStGR1) and two flanking low copy genes Ca-02398 (cytC) and Ca-02400 (50 ORF) are marked by ovals. Location of oligonucleotides used in PCR amplification assays from the vicinity of CaStGR1 and falling within the large deletion are marked by gray arrows, and those from the deletion spanning amplification PCR are marked by blue arrows.

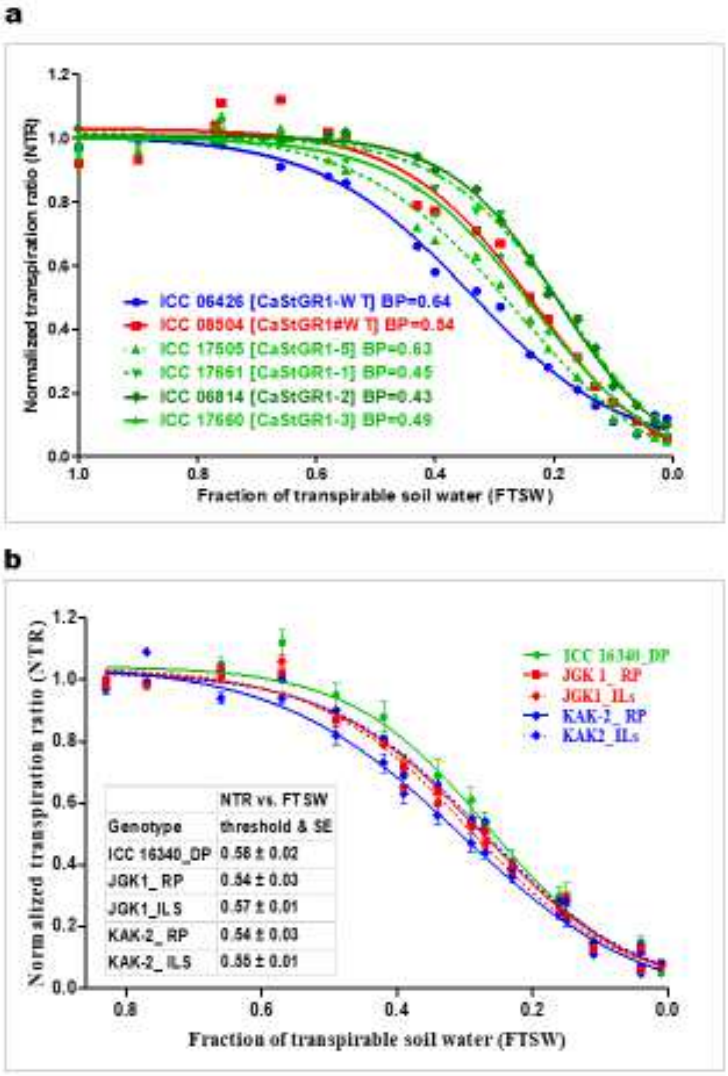

Fig. 2.3 (a) Normalized transpiration ratio (NTR) versus fraction of transpirable soil water (FTSW) of chickpea genotypes differed in deletion of CaStGR1gene segments [ICC 08504-CaStGR-1-#Wild type (filled square with solid red line); ICC 06426-CaStGR-1-Wild type (filled round with solid blue line); ICC 17505-CaStGR-1-5 (filled upward triangle with dashed green line); ICC 17661-CaStGR-1-1 (filled down-word triangle with dashed green line); ICC 06814-CaStGR-1-2 (filled diamond with solid green line) and ICC 17660-CaStGR-1-3 (open round with solid green line)] exposed to progressive drying soil under glasshouse conditions. During detached leaf green assay, ICC 08504-CaStGR-1-#Wild type showed yellow colour in all leaflets fully. By contrast, ICC 06426-CaStGR-1-Wild type showed semi-green colour leaflets. Genotypes with CaStGR1-1 (ICC 17661), CaStGR1-2 (ICC 06814), CaStGR1-3 (ICC 17660), and CaStGR1-5 (ICC 17505) showed completely green colour in all the leaflet during detached leaf green assay. Values are transpiration data of five replicated plants for each genotype at each FTSW condition. The FTSW thresholds where transpiration initiated its decline were calculated with a plateau regression procedure from SAS. The regression lines of the relationships between NTR

and FTSW were drawn by fitting NTR to FTSW data above and below the respective threshold for transpiration decline in each genotype with GraphPad Prism. The FTSW breakpoint (BP) are displayed in the figures. (b) Normalized transpiration ratio (NTR) versus fraction of transpirable soil water (FTSW) of stay green chickpea introgression lines (ILs) with different genetic background [stay green donor parent (DP) ICC 16,340 parent (RP) KAK2 (diamond with solid blue line); KAK2 background introgression lines KAK2-ILs (diamond with dashed red line)] exposed to progressive drying soil under glasshouse conditions. Values are transpiration data of five replicated plants for each genotype at each FTSW condition. The FTSW thresholds where transpiration initiated its decline were calculated with a plateau regression procedure from SAS. The regression lines of the relationships between NTR and FTSW were drawn by fitting NTR to FTSW data above and below the respective threshold for transpiration decline in tested genotype with GraphPad Prism. The FTSW breakpoint (BP) and their confidence intervals of regressions are displayed in the figures.

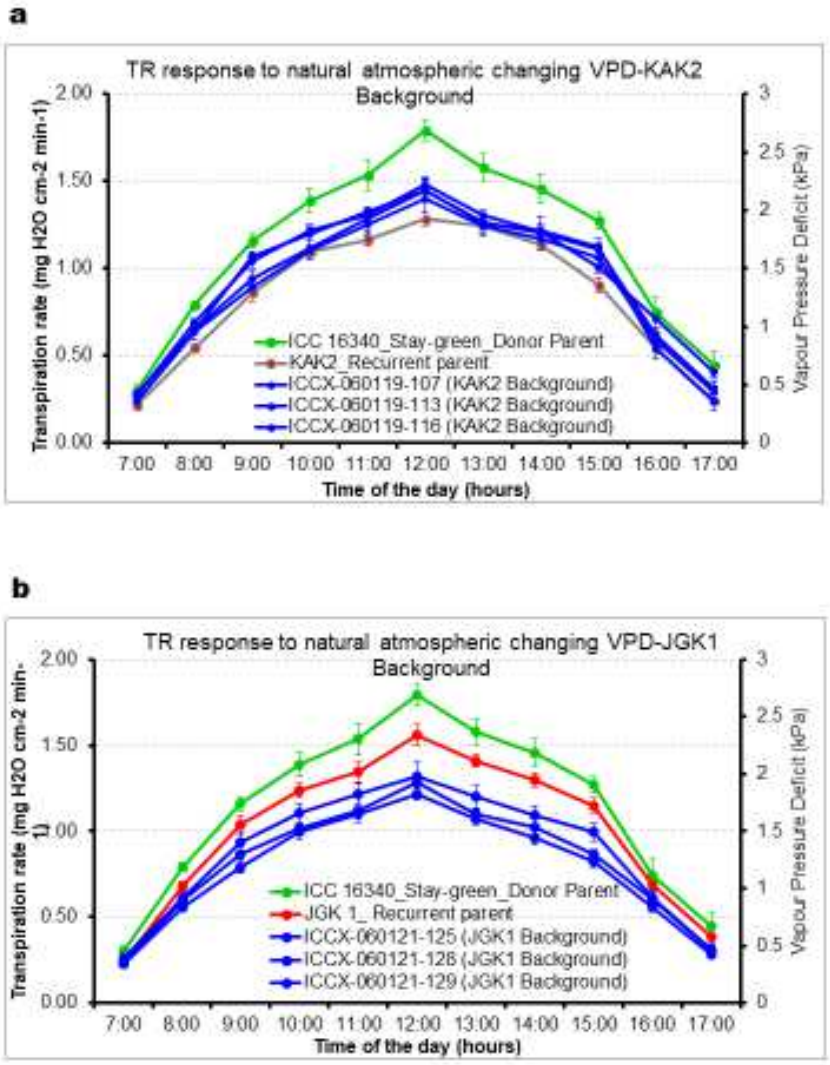

Fig. 2.4 Transpiration rates (TR) of stay green chickpea introgression lines (ILs) with different genetic backgrounds of KAK2 elite cultivar (a), and 4JGK1 elite cultivar (b). Stay green donor parent (DP) ICC 16,340 (round with solid green line); Recurrent parent (RP) KAK2 (round with solid red line); KAK2 background introgression lines ICCX-060119-107, ICCX-060119-113, ICCX-060119-116 and ICCX-060119-123 (round with solid blue line); Recurrent parent (RP) JGK1 (round with solid red line); JGK1 background introgression lines ICCX-060121-125, ICCX-060121-128 and ICCX-060121-129 (round with solid blue line)] are response to natural changing in the atmospheric vapour pressure deficit (VPD) cycle. TRs were measured on well-watered plants grown in the glasshouse, which were temporarily transferred to outdoor conditions. There, plants were exposed to natural changing atmospheric VPD. TR and VPD data were used to draw a segmental or a single linear regression for all tested genotypes. Each data points represents the means (± SE) of eight replicates per genotype.

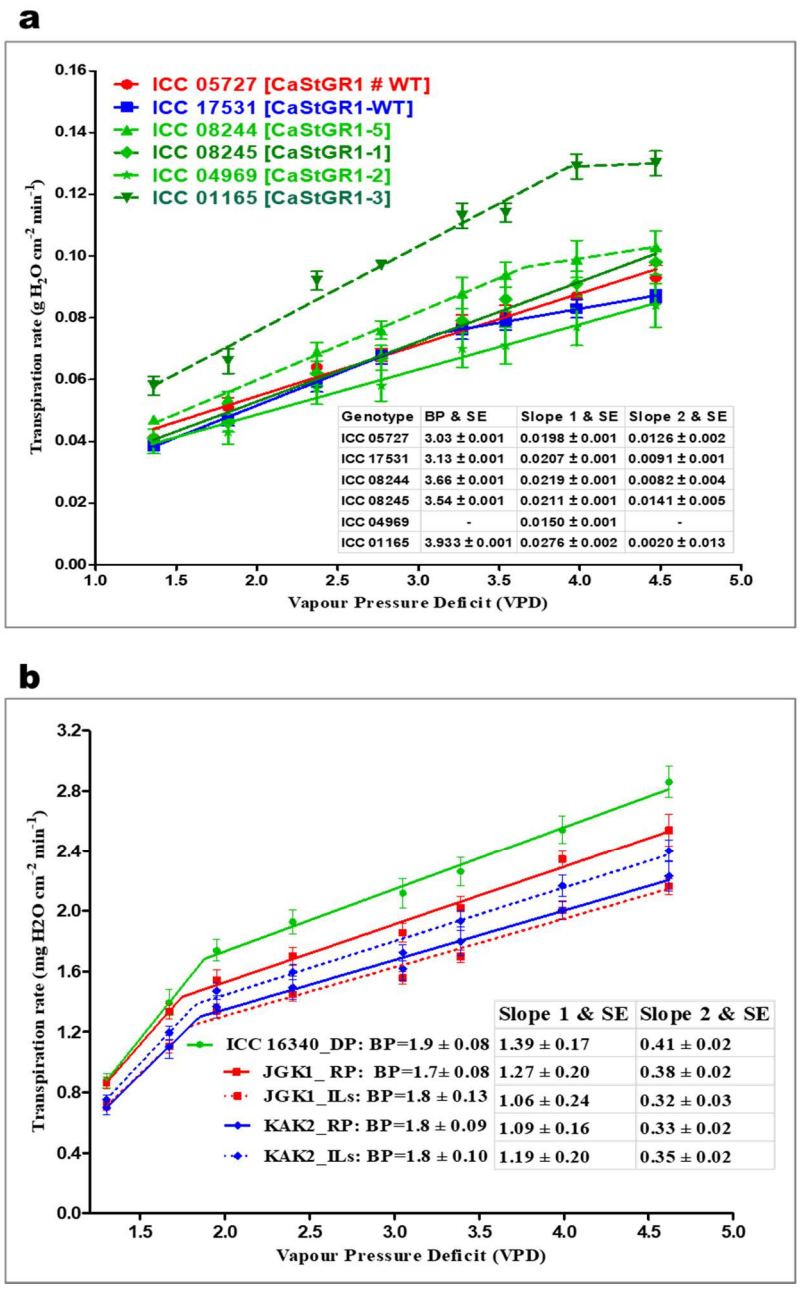

Fig. 2.5 (b) Transpiration rates (TR) of six selected chickpea genotypes differed in deletion of CaStGR1gene segments [ICC 05727-CaStGR-1-#Wild type (round with solid red line); ICC 17531-CaStGR-1-Wild type (square with solid pink line); ICC 08244-CaStGR-1-5 (upward triangle with solid green line); ICC 08245-CaStGR-1-1 (diamond with solid blue line), ICC 04969-CaStGR-1-2 (star with solid orange line) and ICC 01165-CaStGR-1-3 (downward triangle with solid pink line)] in response to increasing VPD. During detached leaf green assay, ICC 05727-CaStGR-1-#Wild type showed yellow colour in all leaflets fully. By contrast, ICC 17531-CaStGR-1-Wild type showed semi-green colour leaflets. Genotypes with CaStGR1-1 (ICC 08245), CaStGR1-2 (ICC 04969), CaStGR1-3 (ICC 01165), and CaStGR11-5 (ICC 08244) showed completely green colour in all the leaflet

during detached leaf green assay. TRs were measured on well-watered plants grown in the glasshouse, which were temporarily transferred to a growth chamber with control over temperature and relative humidity. There, plants were exposed to increasing VPD, set by modifying temperature and humidity. TR data are the mean of five replicate plants, computed hourly at each of the eight VPD levels. Data were used to draw a segmental or a single linear regression for all tested genotypes. Each data points represents the means (± SE) of five replicates per genotype. The slopes and breakpoint (BP) of regressions are displayed in the figures. (b) Transpiration rates (TR) of stay green chickpea introgression lines (ILs) with different genetic background [stay green donor parent (DP) ICC 16,340 (square with solid green line); Recurrent parent (RP) JGK1 (square with solid red line); JGK1 background introgression lines JGK1-ILs (square with dashed red line); Recurrent parent (RP) KAK2 (diamond with solid blue line); KAK2 background introgression lines KAK2-ILs (diamond with dashed red line)] are response to increasing VPD. TRs were measured on well-watered plants Figure 5. (a) Transpiration rates (TR) of six selected chickpea genotypes differed in deletion of CaStGR1gene segments [ICC 05727-CaStGR-1-#Wild type (round with solid red line); ICC 17531-CaStGR-1-Wild type (square with solid pink line); ICC 08244-CaStGR-1-5 (upward triangle with solid green line); ICC 08245-CaStGR-1-1 (diamond with solid blue line), ICC 04969-CaStGR-1-2 (star with solid orange line) and ICC 01165-CaStGR-1-3 (downward triangle with solid pink line)] in response to increasing VPD. During detached leaf green assay, ICC 05727-CaStGR-1-#Wild type showed yellow colour in all leaflets fully. By contrast, ICC 17531-CaStGR-1-Wild type showed semi-green colour leaflets. Genotypes with CaStGR1-1 (ICC 08245), CaStGR1-2 (ICC 04969), CaStGR1-3 (ICC 01165), and CaStGR11-5 (ICC 08244) showed completely green colour in all the leaflet during detached leaf green assay. TRs were measured on well-watered plants grown in the glasshouse, which were temporarily transferred to a growth chamber with control over temperature and relative humidity. There, plants were exposed to increasing VPD, set by modifying temperature and humidity. TR data are the mean of five replicate plants, computed hourly at each of the eight VPD levels. Data were used to draw a segmental or a single linear regression for all tested genotypes. Each data points represents the means (± SE) of five replicates per genotype. The slopes and breakpoint (BP) of regressions are displayed in the figures. (b) Transpiration rates (TR) of stay green chickpea introgression lines (ILs) with different genetic background [stay green donor parent (DP) ICC 16,340 (square with solid green line); Recurrent parent (RP) JGK1 (square with solid red line); JGK1 background introgression lines JGK1-ILs (square with dashed red line); Recurrent parent (RP) KAK2 (diamond with solid blue line); KAK2 background introgression lines KAK2-ILs (diamond with dashed red line)] are response to increasing VPD. TRs were measured on well-watered plants grown in the glasshouse, which were temporarily transferred to a growth chamber with control over temperature and relative humidity. There, plants were exposed to increasing VPD, set by modifying temperature and humidity. Data were used to draw a segmental or a single linear regression for all tested genotypes. Each data points represents the means (± SE) of eight replicates per genotype. The slopes and breakpoint (BP) of regressions are displayed in the figures.

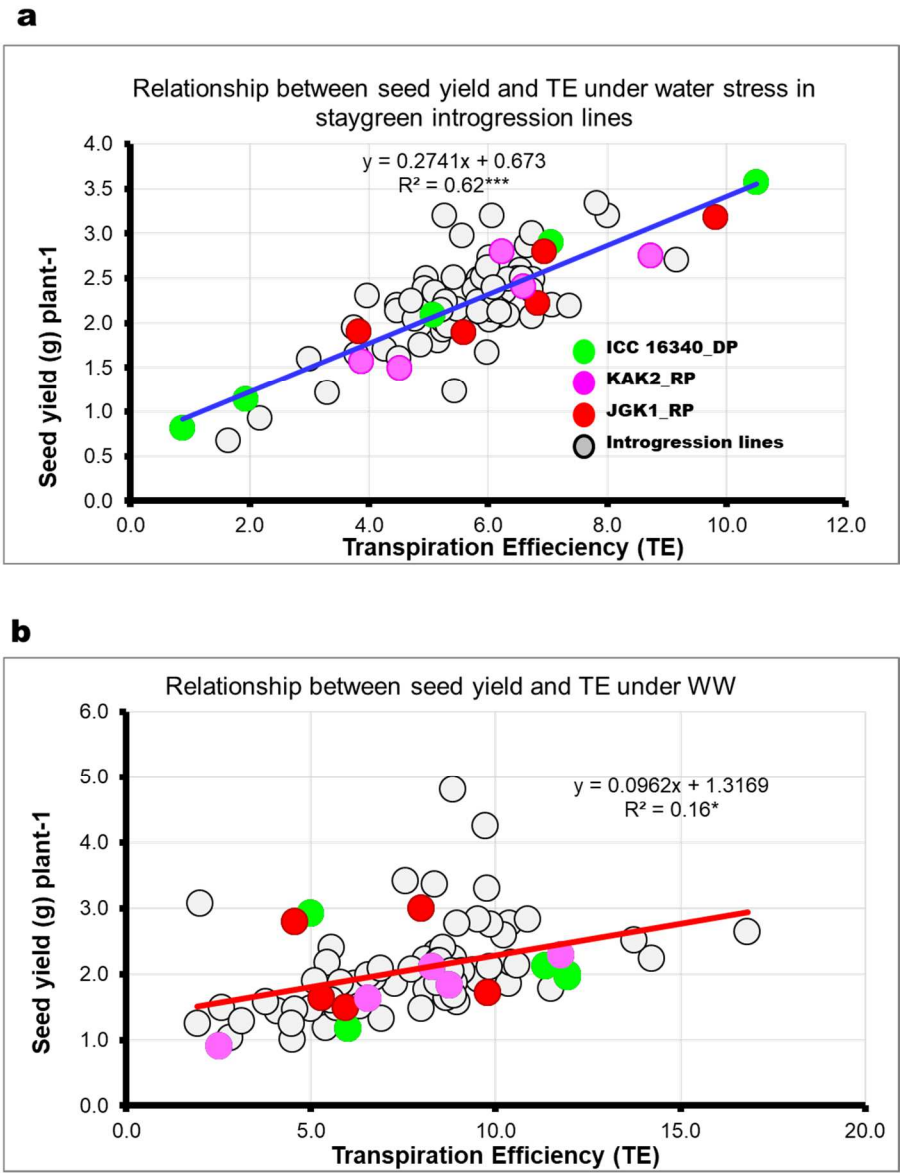

Fig. 2.6 Relationships between seed yield and transpiration efficiency (TE) under (a) water stressed 28 (WS) and (b) well watered conditions (WW) in stay green chickpea genotypes grown in the PVC 29 cylinders (Lysimetric facility). The data used for these regression analyses are replicated data, 30 obtained under WS and WW conditions. For each genotype, five replicates data points were used to 31 draw the linear regressions. The stay green donor parent (ICC 16340) data are represented in green 32 colour, recurrent parent (JGK1) data are represented in red colour, recurrent parent (KAK2) data are 33 represented in pink colour and introgression lines (ILs) are represented in grey colour. The slopes and R2 of regressions are displayed in the figures. R2 34 values with * and *** (astric) symbols are 35 significantly different at $p < 0.05$ and $p < 0.001$.

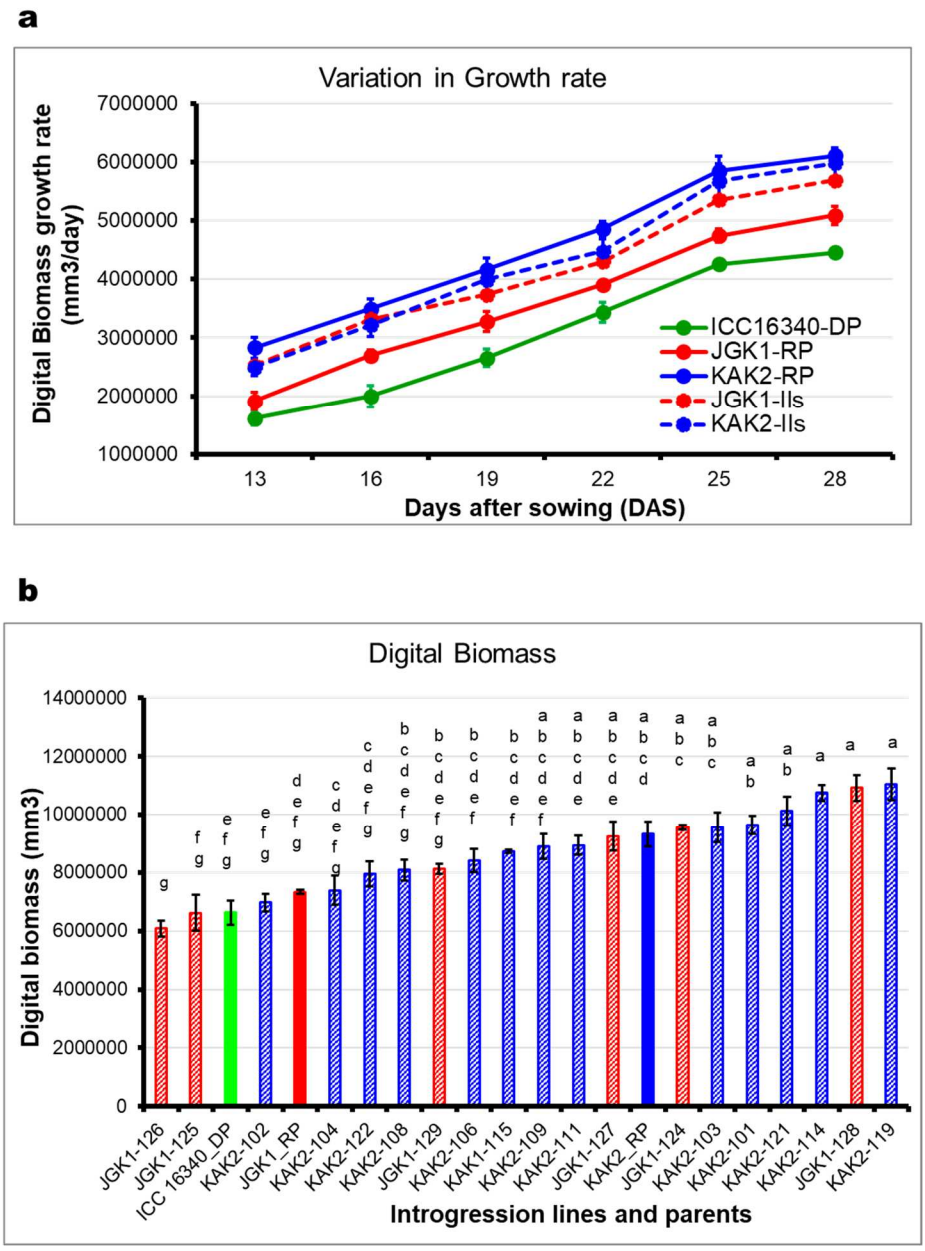

Fig. 2.7 (a) Growth rate variation in digital biomass of stay green chickpea introgression lines (ILs) 50 with different genetic background [stay green donor parent (DP) ICC 16,340 (round with solid green 51 line); Recurrent parent (RP) JGK1 (round with solid red line); JGK1 background introgression lines 52 JGK1-ILs (round with dashed red line); Recurrent parent (RP) KAK2 (round with solid blue line); 53 KAK2 background introgression lines KAK2-ILs (round with dashed blue line)] are measured by 54 LeasyScan phenotyping platform. Each data point represents the means (± SE) of four replicates per 55 genotype. Data were used to draw a line graph for all tested genotypes. (b.) Variation in digital 56 biomass of stay green chickpea introgression lines (ILs) with different genetic background

[stay 57 green donor parent (DP) ICC 16,340 (bar filled with solid green colour); Recurrent parent (RP) JGK1 58 (bar filled with solid red colour); JGK1 background introgression lines JGK1-ILs (bar crossed lines 59 with red colour); Recurrent parent (RP) KAK2 (bar filled with solid blue colour); KAK2 background 60 introgression lines KAK2-ILs (bar crossed lines with blue colour)] are measured by LeasyScan 61 phenotyping platform. Each data points represents the means (± SE) of four replicates per genotype. 62 Data were used to draw a bar graph for all tested genotypes. Bars with different letters are aignificantly different ($p<0.05$).

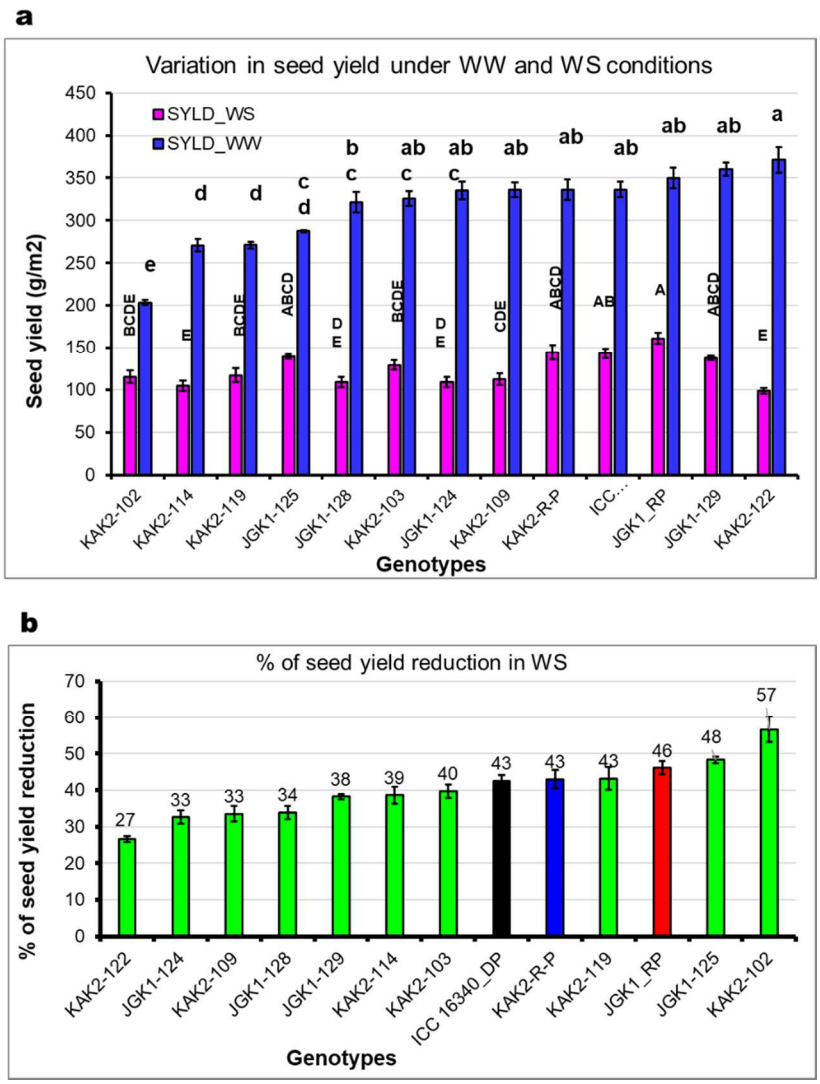

Fig. 2.8 (a) Variation in seed yield under well water (bar filled with blue colour) and water stress (bar filled with pink colour) conditions. The data used for these bar graphs are mean data, obtained under well-watered (WW) and water stress (WS) conditions. For each genotype, three replicates data points were used to draw the bar graph. Bars with different capital letters (well-watered—WW) and small letters (water stressed—WS) alphabets are significantly different ($p < 0.05$) and same letters represents non-significant. (b) Percentage of seed yield reduction under water stress (WS) conditions. The data used for these bar graph are mean data, obtained from well watered seed yield data were normalised against water-stressed seed yield data and then seed yield reduction values are presented in percentage. The data of stay green donor parent ICC 16,340 (bar filled with black colour); recurrent parent-JGK1 (bar filled with red colour); recurrent parent-KAK2 (bar filled with blue colour); stay-green introgression lines from both JGK1 and KAK2 genetic background–ILS (bar filled with green colour).

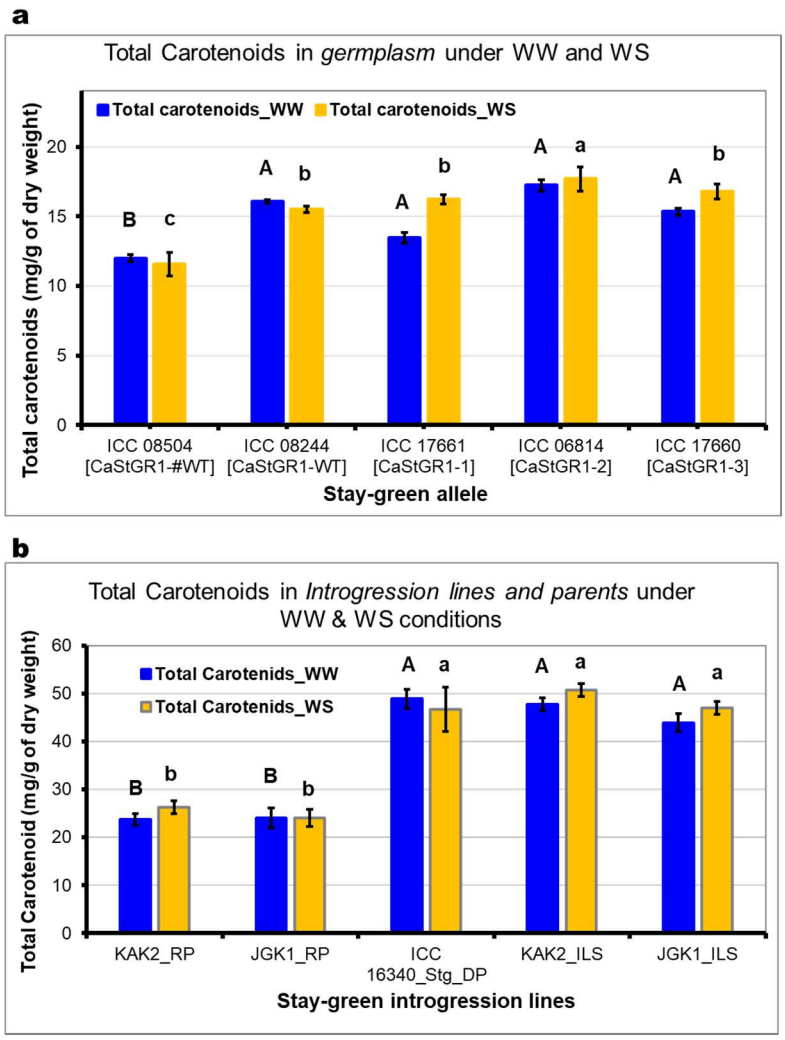

Fig.2.9 Variation in (a) seed total carotenoids content in germplasm [ICC 08,504 (CaStGR1-#WT), ICC 08,244 (CaStGR1-WT), ICC 17,661 (CaStGR1-1), ICC 06,814 (CaStGR1-2) and ICC 17,660 (CaStGR1-3)] and (b) stay green chickpea introgression lines (ILs) with different genetic background under well-watered (WW) and water-stressed (WS) conditions. During detached leaf green assay, ICC 08504-CaStGR-1-#Wild type showed yellow colour fully in all leaflets. By contrast, ICC 08244-CaStGR-1-Wild type showed semi-green colour leaflets. Genotypes with CaStGR1-1 (ICC 17661), CaStGR1-2 (ICC 06814) and CaStGR1-3 (ICC 17660) showed completely green colour in all the leaflet during detached leaf green assay In both graph (a) and (b), closed bars represents WW and open bars are represents WS. Each data points represents the means (± SE) of five replicates per genotype. Data were used to draw a bar graph for all tested genotypes. Bars with different capital letters (well water-WW) and small letters (water stressed-WS) alphabets are significantly different ($p < 0.05$) and same letters represents non-significant

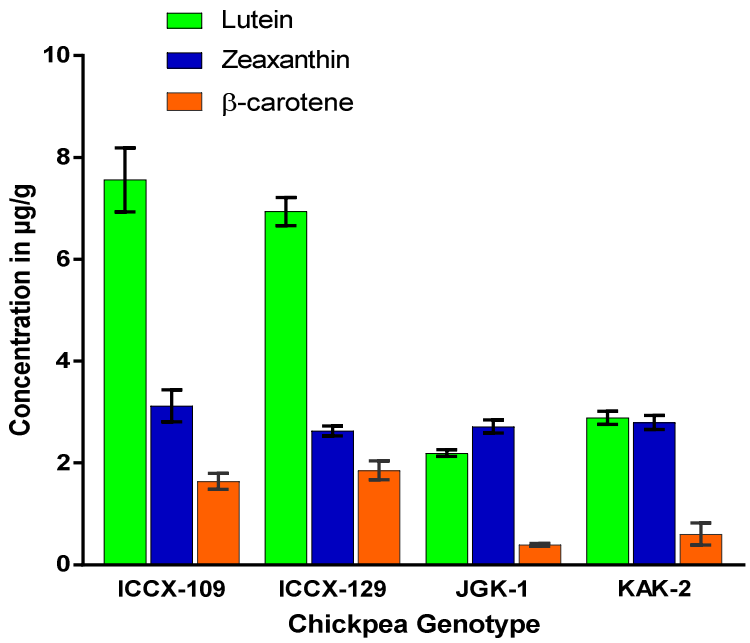

Fig. 2.10 Variation in different carotenoids content (Lutein, Zeaxanthin and beta carotene) in seeds of stay green chickpea introgression lines (ILs) with different genetic background [ICCX-109 (KAK2 genetic background and ICCX-129 (JGK1 genetic background) and their recurrent parents (JGK1 and KAK2). The lutein pigment data are represents in light grey colour bars; Zeaxanthin pigment data are represents in black colour bars and beta carotene pigment data are represents in dark grey colour bars. Each data points represents the means (± SE) of three replicates per genotype.

CHAPTER 3: THE IMPACT OF DOMESTICATION ON ABOVE- AND BELOW- GROUND TRAIT RESPONSES TO NITROGEN FERTILIZATION IN WILD AND CULTIVATED GENOTYPES OF CHICKPEA

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3.2 Abstract

Despite the importance of crop responses to low-fertility conditions, few studies have examined the extent to which domestication may have limited crop responses to low-fertility environments in above- and belowground traits. To this end, studies that have addressed this topic are usually limited in that few wild accessions are used and therefore do not fully capture the genotypic and phenotypic diversity of wild relatives. To examine how domestication has affected the response of above- and belowground agronomic traits, we measured root and leaf functional traits in an extensive set of wild and domesticated chickpea accessions grown in low and high nitrogen environments. The wild accessions used in this study broadly captures the genetic and phenotypic diversity of domesticated chickpea's (*Cicer arietinum)* closest compatible wild relative (*C. reticulatum)*. Our results revealed similar responses to lower nitrogen availability among wild and domesticated chickpea for both above- and below-ground functional traits, with limited evidence of canalization in domesticated chickpea. However, when taking into account specific accessions, we found significant two-way interactions between nitrogen availability and domestication history for several functional traits: specific root length, root density, aboveground biomass, and water use efficiency. Our results suggest that domestication dampened the variation in response type of cultivated chickpea to higher nitrogen environments for below- and aboveground traits in comparison to wild chickpea. In addition, our findings document substantial variation between accessions, particularly in the wild germplasm; thus, highlighting the need for a greater number of wild accessions used in domestication studies.

3.3 Introduction

The practice of artificial selection on organismal traits was a critical innovation in human history that allowed for the rapid directional modification of traits in plants and animals. In crops like legumes, humans primarily selected for traits such as pod indehiscence, reduced seed dormancy, and yield (e.g., Gross and Olsen 2010; Meyer et al. 2012; Olsen and Wendel 2013; Smýkal et al. 2018). However, selection to modify one trait can often lead to a modification in other traits due to trait covariation and underlying genetic linkage (Lande and Arnold 1983; Price and Langen 1992). The evolution of traits through the inadvertent selection of correlated traits and genetic linkage are well documented in a variety of species (Rauw et al. 1998; Kingsolver et al. 2001; Hoekstra et al. 2001; Hereford et al. 2004; Kingsolver and Pfennig 2007). In crops, similar patterns of correlated selection combined with population bottlenecks during domestication may have unintentionally altered non-target traits, potentially canalizing crop responses to different environmental conditions (Flatt, 2005; Morrell et al. 2011; Meyer et al. 2012; Smýkal et al. 2018; Gaut et al. 2018; Lye and Purugganan 2019). Understanding the degree to which domestication has canalized or otherwise altered plant traits, and the ability of plants to respond to low fertility environments can aid agricultural programs to combat food insecurity in a changing global climate.

Domesticated plants typically exhibit exaggerated phenotypic traits (such as bigger seeds/fruits, reduced seed dormancy, altered plant height, apical dominance/reduced branching, seed shattering/fruit abscission, and a loss of vernalization) compared to their wild ancestors - a phenomenon commonly known as a domestication syndrome (DS) (Hammer 1984; Lenser and Theißen 2013). Domestication syndromes are an example of

strong human-imposed selection rapidly shifting a common set of traits in a number of crop species that Nikolai Vavilov termed the 'homologous series of variation' (Smartt 1990), which in some cases have a shared or similar genetic basis (e.g., Meyer et al. 2012; Ogutcen et al. 2018). Although many comparative studies have demonstrated how artificial selection can lead to marked decreases in genomic and phenotypic variation in domesticated plants compared to wild relatives (e.g., Morrell et al., 2011; (Olsen and Wendel 2013; Gaut et al. 2018; Lye and Purugganan 2019; Hufford et al. 2019), the majority of comparative research of phenotypes has focused on the impacts of domestication on aboveground agronomic traits such as seed size or shattering (e.g., Milla et al. 2015; Smýkal et al. 2018; Ogutcen et al. 2018). Relatively few studies have examined the potentially canalizing effects of domestication on belowground functional traits such as root architecture and root-soil-nutrient dynamics (e.g., Bulgarelli et al. 2015; Milla et al. 2015; Pérez-Jaramillo et al. 2016). Even fewer have taken a whole-plant approach to understand the impact of domestication on above- and below- ground traits in tandem; thus, limiting our understanding of how domestication may have impacted plant function. Furthermore, most studies assessing the effects of domestication on crops have utilized very small numbers of genotypes of wild relatives, limiting the power and potential to extrapolate from these comparisons.

Although crop wild relatives have increased the economic value of many crops through disease resistance and other important traits (e.g., Price waterhouse cooper LLC, 2014; Costanza et al. 1997), the ecology of crop wild relatives is generally poorly understood (Warschefsky et al. 2014). Many crop wild relatives are found in environments with limited water availability and nutrient-poor soils compared to their domesticated

counterparts that occur in agricultural farms (e.g., McKey et al. 2012; Grossman and Rice 2012; Milla et al. 2015). However, these wild habitats are heterogeneous and likely to maintain phenotypic plasticity, in contrast to trait canalization (*sensu* Flatt, 2005). As a result, a shift to fertile environments during domestication (i.e., as humans often initially cultivated richer valley soils, and learned to till soils and fertilize crops with animal waste) may have relaxed selective pressures on plant functional traits that impact resource acquisition like carbon, nitrogen, and water uptake from nutrient-poor soils and/or canalized responses under high fertility conditions (Grossman and Rice 2012; Martin and Isaac 2015). Canalization of nutrient uptake traits under nutrient-rich environments in cultivated crop lineages could lead to poorer performance than ancestral wild populations in nutrient-limiting environments from erosion of genes for these traits in cultivated gene pools. This would result in a reduced capacity to grow in low nutrient conditions, such as those typical in many small-holder farming systems in the developing world, for farmers restricted to marginal soils, and to some organic production systems.

The impacts of domestication on belowground traits may be particularly pronounced for crops with complex soil interactions such as legumes. A recent study suggests the domestication of common bean (*Phaseolus vulgaris*), for which domestication and post-domestication selection by humans has focused on fruit yield and size, has also resulted in shifts in traits critical to soil interactions and nutrient dynamics including root microbiome composition, increased specific root length (SPL), and decreased root density (Pérez-Jaramillo et al. 2017). Despite the impact of domestication on agronomic traits, a broad set of root functional traits remain unexplored for most of the world's most economically important crop species. For example, chickpea (*Cicer arietinum*) is the

second most important grain legume globally, and the leading legume in South Asia (Fao et al. 2017). The need for such studies in economically important crop species such as chickpea is more urgent than ever, with reductions in rainfall and soil fertility predicted to result in decreased yields in several food-insecure areas like India, Ethiopia, and Turkey, where chickpea is a key source of nutritional security and a cash crop (Singh et al. 2014; Ahmed et al. 2016). Therefore, understanding the degree to which domestication has impacted plant traits, and the ability of plants and traits to respond to new environments, is critical to adapting agricultural programs in a changing climate.

Chickpea is an ideal system to address the impacts of domestication on above- and belowground phenotypes because it is one of the earliest domesticated crops with a well-studied domestication history (Redden and Berger 2007; von Wettberg et al. 2018). Chickpea was domesticated during the Neolithic period 12,000 years ago in the nutrient-poor arid mountain ridges of southeast Anatolia and has undergone four evolutionary genetic bottlenecks that have severely reduced genomic and phenotypic variation (Abbo et al. 2003; Redden and Berger 2007; von Wettberg et al. 2018). In line with other studies on the impact of domestication on the phenotypic plasticity for resource acquisition (Grossman and Rice 2012; Martin and Isaac 2015), it is possible that selection practices on chickpea in high fertility environments led to a canalized response (loss of plasticity) in above- and belowground traits related to resource acquisition such as carbon, nitrogen, and water use relative to its wild relatives which are generally adapted to low nitrogen environments.

To understand how domestication affected above- and belowground agronomic traits, resource-use efficiency, and adaptive capacity in crops, we assembled a uniquely large collection of wild chickpeas from southeastern Turkey, providing sufficient numbers of genetically distinct wild genotypes to examine differentiation in above- and belowground phenotypes between cultivated crops and their wild relatives (von Wettberg et al. 2018). We grew wild and domesticated chickpea accessions in low and high nitrogen concentrations and measured root and leaf functional traits. We hypothesized that if domestication for typical agronomic traits has resulted in inadvertent selection in other functional traits due to cultivation in higher fertility environments that are typical of agriculture, then **1)** wild accessions will have traits consistent with greater performance and resource use efficiency in low nutrient conditions compared to domesticated accessions, and **2)** domesticated accessions will exhibit lower phenotypic plasticity in root and leaf functional traits.

3.4 Methods and Materials

3.4.1 Plant germplasm used

Twenty-seven genetically diverse accessions of chickpea were used in this study (Table 1). Six accessions: CDC Frontier, ICC16207, Gokce, Dwelley, Myles, and UC15 are cultivars originating from four countries: United States, Canada, Turkey, and India. These accessions were selected because they represent both chickpea market types, Desi and Kabuli (Penmetsa et al. 2016), and are widely grown in their native countries. The remaining 21 accessions are wild chickpea lines systematically collected from different regions of Turkey, the native range of wild chickpea (von Wettberg et al. 2018). These

accessions were selected to maximize genetic and native environmental differences in the material to capture as much wild diversity as possible.

3.4.2 Experimental Design

All accessions were grown in a shade house at Fairchild Tropical Botanic Garden in Coral Gables, Florida, from Dec-2016 to Mar-2017. Average day and night temperature during this period ranged between 27ºC and 16ºC, and average monthly rainfall was 5 cm (USclimatedata.com). Seeds of each accession were planted in 11-liter pots containing 8 liters of a mixture of sand and coconut coir. This mixture was used as a planting media to minimize the nitrogen present before preparation. Plants were watered every 48 hours by an automatic sprinkler system.

Eight replicates of each accession were subjected to two different nitrogen treatments: 1 ppm (2.362 mg N source/L planting media) and 100 ppm (238506.2 mg/L). ESN Polymer Coated Urea (Agrium U.S. Inc.), a slow-release nitrogen pellet was used as the nitrogen source. These treatments were chosen to represent generally nutrient poor conditions in the wild, and a typical nitrogen level found in an agricultural field setting, respectively. To make sure other nutrients were not limiting for chickpea growth, all pots received 2.40 mg/L Phosphorus (P) as $Al(PO_3)_3$, 470.8 mg/L Calcium (Ca) as $CaSO_4 \cdot 2H_2O$, 507.8 mg/L Magnesium (Mg) as $MgSO_4 \cdot 7H_2O$, 2.598 mg/L Copper (Cu) as $CuSO_4 \cdot 5H_2O$, 5.401 mg/L Zinc as $ZnSO_4 \cdot 7H_2O$, 22.96 mg/L Manganese (Mn) as $MnSO_4 \cdot H_20$, 2.499 mg/L Boron (B) as $Na_2B_4O_7 \cdot 10H_2O$[/] and 0.119 mg/L Molybdenum (Mo) as $Na_2MoO_4 \cdot 2H_2O$. Plants were grown in the absence of rhizobial symbionts, as evidence suggests that wild and cultivated chickpea differ in symbiont preference

(Greenlon *et al*., unpublished, Greenlon et al. 2019), and rhizobial symbionts differ in their tolerance of different soils (Alford *et al*., unpublished, Greenlon et al. 2019). All pots were randomly arranged in a grid in the shade house.

3.4.3 Gas-exchange

Gas-exchange measurements were performed on mature leaflets for 6-8 individuals per genotype using the LI-6400 infrared gas analyzer (Li-6400, Li-Cor Inc., NE, USA). Chamber conditions were set to 1300 μmol PAR and CO_2 concentration of 400 ppm. The block temperature was set to 28°C achieving an average temperature of 28.91°C (+/- 1.35 °C) and the vapor pressure deficit (VPD) was 1.38 kPa (+/- 0.37 kPa). After gas exchange rates had stabilized (≥ 6 min), net photosynthetic rates (A_N) and stomatal conductance (g_S) were recorded. The leaf area was corrected using digital photographs of the leaf material that was inside the chamber using ImageJ (Wayne Rasband/NIH, Bethesda, MD, USA). Gas-exchange measurements were taken between 0800 and 1300 hours.

3.4.4 Stable Isotope Chemistry

The leaflets used for gas-exchange were cut and digitally photographed in the field (for later analysis of specific leaf area) and then placed into coin envelopes and stored in a drying oven at 75°C for at least 72 hours before being weighed. Leaflet area was calculated in ImageJ (Wayne Rasband/NIH, Bethesda, MD, USA), and specific leaf area (SLA) was calculated from the ratio of fresh area (cm^2) and dry mass (g). The dried samples were then run through a Carlo Erba NC2500 elemental analyzer (CE Instruments Ltd. England, UK) in tandem with a Thermo Delta V Stable Isotope Mass Spectrometer (Thermo Fisher

Scientific Inc. Waltham, MA, USA) at the Cornell University isotope lab (COIL) to measure elemental chemistry i.e. δ13C and %N.

3.4.5 Root and Canopy Morphology

Above- and belowground plant biomass was harvested twelve weeks after sowing. Aboveground biomass was defined as all living biomass above the soil. A subset of wild and domesticated replicates of each accession for each treatment (1 ppm and 100 ppm) were randomly selected for leaf area measurements. All leaves of selected plants were removed, laminated, and scanned at 1200 dpi using an Epson Perfection V700 scanner (Epson America, Long Beach CA). Lamination prevented folding of leaves during scanning and allowed more measurements to be taken by slowing down wilting. For the remaining plants, aboveground biomass was placed in a drying oven for 24 hours, after which the dry mass was recorded using an analytical balance (Mettler Toledo ME103TE, Columbus, OH, USA). All belowground biomass was carefully separated from the soil, cleaned with deionized water, and scanned for image analysis. The samples were then dried and weighed as described above. The image analysis system, WinRHIZO (version *Arabidopsis*) was used to calculate root length [i.e. RL], average root diameter [i.e. RD], root surface area [i.e. RSA], root volume [i.e. RV], and leaf area [i.e. LA] from root and leaf scans (Regent Instruments, Quebec City, Quebec, Canada). Specific root length and root density were calculated by dividing root length (cm) by belowground biomass (g) and belowground biomass (g) by root volume (cm^3), respectively.

3.4.6 Data Analysis

A nested generalized linear mixed model (GLMM) (Bates et al. 2015; Kuznetsova et al. 2017) was used to test for significant differences for all measurements between treatments (1 ppm and 100 ppm), history (Wild or Domesticated), and accessions (i.e., genotype). Treatment was used as a fixed factor, while accession, a random factor, was nested into history, a fixed factor, making the whole term random.

To understand if responses to soil nitrogen are dependent on domestication history, we examined the history by treatment interactions of our GLMM for all traits. To better understand the direction and intensity of trait responses, we further calculated the relative distance plasticity index (RDPI) for traits with significant history by treatment interactions (Valladares et al. 2006). Specifically, we calculated RDPI for specific root length (x ^ lambda-transformed), root density (log-transformed), water-use efficiency (x ^ lambda-transformed), aboveground biomass (log-transformed), canopy level photosynthesis (log-transformed), and stomatal conductance (x ^ lambda-transformed) using the Plasticity R package (Ameztegui 2017). Lastly, we correlated specific root length (x ^ lambda-transformed), root density (log-transformed), and water-use efficiency (x ^ lambda-transformed) with aboveground biomass (log-transformed) to test whether plant plasticity may affect plant fitness. We used plant biomass as a fitness indicator rather than seed set because we harvested before flowering to capture intact root systems. All statistical analyses were performed in R (www.r-project.org).

3.5 Results

3.5.1 Below- and aboveground traits in low nitrogen environments

At the lower nitrogen level (1 ppm), the majority of belowground morphological traits among domesticated and wild chickpea were comparable, with non-significant differences in belowground biomass (t_{292} = 2.099, P = 0.156) (Figure 1a), root length (t_{292} = 2.492, P = 0.063), root density (t_{292} = -2.265, P = 0.109) (Figure 1d), average root diameter (t_{292} = 1.692, P = 0.329), root volume (t_{292} = 1.1305, P = 0.560), and SRL (t_{292} = 1.171, P = 0.646) (Figure 1c), However, domesticated chickpea exhibited significantly higher root surface area than wild chickpea (t_{292} = 2.600, P = 0.048).

Furthermore, we found no significant differences between domesticated and wild chickpea in low nitrogen conditions with respect to aboveground biomass (t_{292} = 1.208, P = 0.622) (Figure 1b), %N (t_{292} = 1.329, P = 0.055), %C (t_{292} = .300, P = 0.991), chlorophyll content index (t_{292} = 1.429, P = 0.482), nitrogen investment into chlorophyll (t_{292} = -.831, P = 0.839), stomatal conductance (t_{83} = .305, P = 0.990), canopy photosynthetic rates (t_{83} = .412, P = 0.976) (Figure 2d), photosynthetic nitrogen-use efficiency (PNUE) (t_{83} = .177, P = 0.998) (Figure 2b), and leaf level photosynthetic rate (Maximum Photosynthetic Rate per area t_{83} = 0.744, P = .879; Maximum Photosynthetic Rate per mass t_{83} = 0.744, P = .879). However, several functional traits varied significantly between wild and domesticated chickpea especially within the low nitrogen treatment, with domesticated chickpea showing higher specific leaf area (t_{83} = -1.282, P = <.001) (Figure 2c), and wild chickpea exhibiting greater water-use efficiency (t_{83} = 2.483, P = .018; Figure 2a), and carbon to nitrogen ratio (t_{292} = -4.487, P = 0.001).

3.5.2 Below- and aboveground traits in high nitrogen environments

Conversely, within the higher nitrogen level (100 ppm), most belowground morphological traits between domesticated and wild chickpea were significantly different. Domesticated chickpea exhibited greater root length (= 1.754, P = 0.298), average root diameter (t_{292} = 3.308, P = 0.006), root volume (t_{292} = 3.988, P = 0.001), and root surface area (t_{292} = 3.822, P = <.001) compared to wild chickpea. SRL (t_{292} = -2.988, P = 0.016) was significantly higher in wild chickpea than in domesticated chickpea (Figure 1c). Domesticated chickpea displayed increased belowground biomass (t_{292} = 1.100, P = 0.411) (Figure 1b) and root density (t_{292} = 1.927, P = 0.319) (Figure 1d) at the higher nitrogen level, but was not significantly different from wild chickpea.

Within the higher nitrogen level (100 ppm), several aboveground functional traits were significantly different between domesticated and wild chickpea. Domesticated chickpea exhibited greater aboveground biomass (t_{292} = 5.347, P = <.001) (Figure 1b), leaf level photosynthetic rate (per area t_{83}= 4.006, P = .001; per mass t_{83} = 4.006, P = .001), canopy photosynthetic rates (t_{83} = 4.715, P = <.001) (Figure 2d), specific leaf area (t_{83} = 5.083, P = <.001) (Figure 2c), photosynthetic nitrogen-use efficiency (PNUE) (t_{83} = 3.278, P = 0.012) (Figure 2b), and stomatal conductance (t_{83} = 3.014, P = 0.018) when compared to wild chickpea. Conversely, domesticated and wild chickpea did not differ in %N (t_{292} = 1.520, P = 0.427), %C (t_{292} = 1.107, P = 0.686),), chlorophyll content index (t_{292} = 0.821, P = 0.845), nitrogen investment into chlorophyll (t_{292} = .383, P = 0.981), water-use efficiency (t_{83} = 1.533, P = .419) (Figure 2a), and C/N ratio (t_{292} = 0.118, P = 0.994) within the higher soil nitrogen treatment (100 ppm).

3.5.3 Phenotypic plasticity in wild and domesticated chickpea

Significant interactions between nitrogen level and domestication history revealed differences in root phenotypic plasticity between wild and domesticated chickpea for root density (f_{292} = 2.953, P = <0.003; Figure 3b) and SRL (f_{292} = 12.568, P = <0.001; Figure 3a)(Supplementary Table S1). As nitrogen levels increased, root density in domesticated chickpea increased (t_{292} = -3.372, P = 0.005), whereas in wild chickpea (t_{292} = 1.519, P = 0.427) root density remained constant across treatments. Conversely, as nitrogen level increased domesticated chickpea significantly reduced SRL (t_{292} = -4.905, P = <0.001), while SRL for wild chickpea remained broadly consistent (t_{292} = -1.177, P = .642) (Figure 1c).

Moreover, significant interactions between nitrogen level and domestication history were present for several aboveground traits, including aboveground biomass (f_{292} = -2.933, P = 0.004), water use efficiency (f_{292} = -4.112, P = 0.004; Figure 3c), C/N ratio (t_{276} = 3.390, P = <0.001), whole canopy photosynthesis (f_{76} = 11.179, P = 0.001; Figure 3d), leaf level photosynthesis (per area, f_{76} = 6.462, P = .013; per mass, *f_{76} = 11.321, P = .001*), and stomatal conductance (f_{76} = 4.137, P = 0.045). Specifically, as nitrogen levels increased, wild chickpea exhibited similar water-use efficiency, while domesticated chickpea increased water-use efficiency in the high nitrogen soil. Thus, both wild (t_{83} = -1.282, P = 0.576) and domesticated (t_{83} = -1.937, P = 0.221) chickpea displayed a canalized response in regards to SLA.

Our analyses of RDPI for traits with significant history by treatment interactions revealed that domesticated chickpea had significantly higher plasticity for aboveground

biomass (t_{2050} = 23.948, P = <0.001), water-use efficiency (t_{1978} = 9.796, P = <0.001), SRL (t_{2128} = 4.301, P = <0.001), and root density (t_{1932} = 14.447, P = <0.001), relative to wild chickpea. However, wild chickpea had significantly higher plasticity for stomatal conductance (t_{769} = -5.464, P = <0.001) and canopy photosynthesis (t_{946} = -4.499, P = <0.001).

Lastly, plant size (i.e. above- or belowground biomass) and plant plasticity (traits that exhibit plasticity) were negatively correlated for wild and domesticated chickpea across both treatments (Supplementary Figure S1). This was indicated by a significant overall negative correlation between plasticity in SRL and aboveground plant biomass (t_{294} = -4.838, P = <0.001, r = -.272; Figure S1a) and a significant overall negative correlation between water-use efficiency and aboveground plant biomass (t_{294} = -2.432, P = .016, r = -.141; Figure S1b). The negative correlation held true for both wild (t_{212} = -2.321 P = .021, r = -.157) and domesticated chickpea (t_{80} = -5.563, P = <0.001, r = -.528) for SRL and aboveground plant biomass across treatments. However, for water-use efficiency and aboveground biomass, domesticated and wild chickpea differed in their correlations; with wild chickpea (t_{212} = -.513 P = .609, r = -.035; Figure S1c) having a non-significant correlation while domesticated chickpea having a strong negative correlation (t_{80} = -4.168 P = <0.001, r = -.422; Figure S1d). Root density and aboveground biomass were not significantly correlated for wild and domesticated chickpea across treatments (t_{294} = -1.298, P = .196, r=-.075).

3.5.4 Substantial variation by ecotype

We found significant variation among accessions for the following belowground traits: belowground biomass (χ^2 (1) = 17.092, P = <0.001), root density (χ^2 (1) = 4.396, P = 0.036) (Figure 3b), root length (χ^2 (1) = 9.987, P = 0.002), average root diameter density (χ^2 (1) = 8.619, P = 0.003), root volume (χ^2 (1) = 7.598, P = 0.005), root surface area (χ^2 (1) = 23.037, P = <0.001), and SRL (χ^2 (1) = 5.298, P = 0.022) (Figure 3a). Additionally, significant response variation for accession was found for several aboveground traits: aboveground biomass (χ^2 (1) = 5.506, P = 0.019), whole canopy photosynthesis (χ^2 (1) = 3.957, P = 0.047) (Figure 3d), leaf level photosynthetic rate (per area χ^2 (1) = 4.203, P = 0.404), water-use efficiency(χ^2 (1) = 26.821, P = <0.001) (Figure 3c), chlorophyll content index (χ^2 (1) = 9.428, p = 0.002), and nitrogen investment into chlorophyll (χ^2 (1) = 12.109, P = <0.001).

3.6 Discussion

Overall, wild and domesticated chickpea had similar phenotypes at low nitrogen concentrations for both belowground and aboveground traits, indicating that domestication has not affected chickpeas response to low nitrogen conditions in the absence of rhizobia. However, significant two-way interactions between nitrogen concentration and history (wild vs. cultivated) for SRL, root density, aboveground biomass, and water use efficiency demonstrated that wild chickpea and domesticated chickpea exhibited differences in their responses to nitrogen. Our results, surprisingly, suggest that wild chickpea had a canalized response for SRL, and limited phenotypic plasticity for most traits except, stomatal conductance and canopy photosynthesis. Both cultivated and wild chickpea exhibited a canalized response for SLA, but SLA was consistently higher for domesticated chickpea at both nitrogen treatments, indicating it is adapted to nutrient rich environments.

Additionally, contrary to one of our primary hypotheses, domesticated chickpea showed greater plasticity than wild chickpea, consistently having the highest average phenotypic plasticity for most traits.

The lower plasticity of many traits in wild chickpea is primarily explained by the substantial accession-by-accession variation within the wild germplasm, which reduced the average phenotypic response to increased nitrogen. This substantial accession-by-accession variation in wild chickpea is not surprising as accessions originate from different environmental conditions (von Wettberg et al., 2018). However, the lack of plasticity and the similar performance and resource use efficiency in low nutrient conditions is surprising, as these are potential mechanisms to increase plant survival in natural environments (reviewed in Ghalambor et al. 2007; Hauvermale and Sanad 2018). Specifically, root plasticity is beneficial for wild plants due to heterogeneous nutrient distribution and limiting nutrients found in natural habitats when compared to agroecosystems (Bennett *et al*., 2005; Paz-González et al. 2000). For instance, for agricultural top-soil, inorganic nitrogen distribution was found to be homogenous (Jackson and Bloom 1990), while nutrient distribution varied significantly in natural sagebrush steppe-habitat (Jackson and Caldwell 1993) and tropical forests (John *et al*., 2007). Furthermore, domesticated crops such as barley (Grossman and Rice 2012), cassava (Ménard et al. 2013), and soybeans (Kiers et al. 2007) have undergone a reduction in phenotypic plasticity which is believed to be due to a reduction in genetic diversity driven by agronomic selection (Sadras et al., 2007) or continuous selection in a more homogenous agricultural environment.

As expected, mean above- and below-ground biomass increased with higher nitrogen levels for both domesticated and wild chickpea. However, for several wild accessions, above- and below-ground biomass decreased or remained relatively the same in higher nitrogen conditions, indicating limited phenotypic plasticity for these traits to nitrogen availability. These results are surprising, as they contradict previous results comparing plasticity in aboveground biomass to nutrient availability in domesticated and wild: chard (*Beta vulgaris* L.), cabbage (*Brassica oleracea DC.*), sunflower *(Helianthus annuus* L.), tomato *(Solanum lycopersicum* L.), durum wheat (*Triticum durum* Desf.), maize (*Zea mays* L.), and pea (*Pisum sativum* L.) (Matesanz and Milla, 2018). Differences between our results and previous findings could stem from the number of accessions used in each study. The limited number of accessions used in previous studies likely were not sufficient to fully capture the phenotypic variation or plasticity present in each crop or wild relative (Krieg et al. 2017).

As nitrogen availability increased, domesticated chickpea accessions reacted uniformly with decreased SRL and increased root density. These results indicate that domesticated chickpea increased root diameter and decreased root length, an expected physiological response to higher nitrogen presence (von Wettberg and Weiner 2003; Callaway et al. 2003). Low SRL and high root density are the predicted root phenotypes for plants in nutrient-rich environments, as these phenotypes are believed to be most efficient when nutrients are abundant (Reich 2014; Kong et al. 2019). Conversely, on average, SRL and root density remained relatively unchanged for wild chickpea in both nitrogen treatments and were not significantly different. However, when taking into account how individual wild accessions reacted to increased nitrogen availability, we

observed variation among accessions in phenotypically plastic responses to nitrogen. With respect to SRL and root density, wild accessions decreased, increased, or remained constant in response to nitrogen availability. These results were surprising as wild bean accessions on average have greater SRL and root density than domesticated accessions (Pérez-Jaramillo et al. 2017). Greater SRL has been hypothesized to provide higher efficiency of nutrient search and uptake, a beneficial phenotype for nutrient 'foraging' in nutrient heterogeneous environments.

Leaf measurements such as leaf-level photosynthesis, stomatal conductance, and canopy level photosynthesis were consistently greater for domesticated chickpea and at both nitrogen levels; however, domestication history was not statistically significant while nitrogen level was (Supplementary Table S1). This is not surprising as the cultivated varieties were likely to have been selected under higher fertility agricultural conditions when compared to those experienced by wild accessions

Domesticated chickpea showed a similar general increase in water-use-efficiency (δ13C), %N in leaves, chlorophyll content, and photosynthetic nitrogen use efficiency (PNUE) to increased nitrogen availability, an expected response to nutrient rich environments (Matesanz and Milla 2018). When focusing in on accession variation, wild chickpea accessions did not respond consistently to increased nitrogen level in regards to water-use-efficiency (δ13C). However, at lower nitrogen conditions, wild accessions displayed similar nitrogen and water use phenotypes, as might be expected from adaptation to low-nitrogen conditions of the native range of wild chickpea in Southeastern Turkey. One of the few measurements that was primarily influenced by domestication history was

SLA, which was not affected by increased nitrogen presence. Domesticated chickpea accessions had consistently higher SLA than wild chickpea (Figure 2; Supplementary Table 1), perhaps as an indirect consequence of selection in domesticated cultivated chickpea for early growth and plant maturity, as evidenced by early phenology of cultivated chickpea (Ortega et al. 2019). Additionally, domesticated chickpea had a slight increase in leaf %C relative to wild accessions, but this was not significant.

Lastly, a limitation to our results is that we performed our study in the absence of symbiotic rhizobia. During root morphology measurement, nodules were rarely found. It was an experimental necessity, as wild and cultivated chickpea differ in their preferred rhizobia and have substantial interactions with soil substrate (Cook, Greenlon et al., unpublished; Greenlon et al., 2019), adding multiple rhizobial strains would make the experiment too complicated to dissect a signal of response to nitrogen fertility. An experiment without rhizobia is a realistic scenario for cases when a crop is grown in new soil, or in a soil that has not had chickpea for over several prior years. We suspect that wild relatives with short dispersal distances may have a greater chance to encounter nearby co-adapted symbionts than their cultivated relatives (Greenlon et al. 2019). When moved beyond their native range or grown in soils lacking a compatible symbiont, wild chickpea may consequently perform more poorly under nutrient limiting conditions. However, when wild chickpea would occur in agricultural conditions, they may on average, experience fertility much higher than in uncultivated habitats. It is also possible that selection may not have been sufficient to canalize responses to low nutrient availability, particularly if there is only a very limited cost to plasticity for root responses to low nutrient availability. The only existing data of which we are aware of is that of Grossman and Rice (2012), who

showed a loss of root plasticity in cultivated barley accessions. An earlier study by Kiers et al. (2007), showed that bred soybean varieties had a reduced capacity to enforce sanctions on low-performing rhizobia, but our study is limited in that we did not examine a broader set of root traits. Conversely, in other crops, the impacts of domestication on crop functional traits remain difficult to predict, especially for belowground traits that have not been systematically studied.

3.6.1 Conclusion

The potentially widespread loss of phenotypic plasticity of crops to low fertility environments, as a consequence of domestication, could be a concern, particularly for farmers working on degraded or marginal soils without access to expensive inputs, or some organic production systems. Here, we find evidence that wild and domesticated chickpea display similar efficient responses to low nitrogen conditions, and canalization of some root traits in wild chickpea. However, when focusing on the accession level, we found significantly more variation in wild chickpea than domesticated chickpea, indicating that wild chickpea is a repository for novel responses to nitrogen conditions. Under Green Revolution agroecological conditions, it is not uncommon for there to be such high levels of added nitrogen in the soil that it results in reduced levels of nodulation in legumes (e.g., Kiers et al. 2007). However, if such excess nitrogen is not present, the loss of phenotypic plasticity is a concern for the performance of crops in more challenging conditions. For a crop like chickpea, which is still largely produced by small-holder farmers as a low or minimal fertilizer input crop in South Asia and East Africa, and that serves a critical food security role in many diets, lost phenotypic plasticity may reduce resilience against climate

change. The genetic bottlenecks that arise from domestication, post-domestication divergence, and the intensive breeding for agronomic traits may have additional, inadvertent effects on unselected belowground traits (e.g., Morrell et al. 2013; Gaut et al. 2018). These inadvertent effects are one of several reasons why large collections of wild relatives with a greater range of adaptive traits or plasticity than in the cultigen, are needed in breeding programs to increase the resilience of our crops within a changing global climate (Warschefsky et al. 2014, Coyne et al., 2020).

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3.8 Tables

Table 3.1 Germplasm with geographic and domestication history information

Germplasm	Species	Geographical Origin	History	Market Type
CDC Frontier	*Cicer arietinum*	Canada	Domesticated	Kabuli
ICC16207	*Cicer arietinum*	India	Domesticated	Desi
Gokce	*Cicer arietinum*	Syria	Domesticated	Kabuli
Dwelley	*Cicer arietinum*	United States	Domesticated	Kabuli
Myles	*Cicer arietinum*	United States	Domesticated	Kabuli
UC 15	*Cicer arietinum*	United States	Domesticated	Kabuli
Bari1 092	*Cicer reticulatum*	Turkey	Wild	Wild
Bari2 072	*Cicer reticulatum*	Turkey	Wild	Wild
Bari3 072n2	*Cicer reticulatum*	Turkey	Wild	Wild
Bari3 100	*Cicer reticulatum*	Turkey	Wild	Wild
Bari3 106	*Cicer reticulatum*	Turkey	Wild	Wild
Besev 075	*Cicer reticulatum*	Turkey	Wild	Wild
Besev 079	*Cicer reticulatum*	Turkey	Wild	Wild
CudiA 152	*Cicer reticulatum*	Turkey	Wild	Wild
CudiB 022C	*Cicer reticulatum*	Turkey	Wild	Wild
Derei 070	*Cicer reticulatum*	Turkey	Wild	Wild
Derei 072	*Cicer reticulatum*	Turkey	Wild	Wild
Egill 065	*Cicer reticulatum*	Turkey	Wild	Wild
Egill 073	*Cicer reticulatum*	Turkey	Wild	Wild
Kalka 064	*Cicer reticulatum*	Turkey	Wild	Wild
Kayat 077	*Cicer reticulatum*	Turkey	Wild	Wild
Kesen 075	*Cicer reticulatum*	Turkey	Wild	Wild
Oyali 084	*Cicer reticulatum*	Turkey	Wild	Wild
Oyali 111	*Cicer reticulatum*	Turkey	Wild	Wild
Sarik 067	*Cicer reticulatum*	Turkey	Wild	Wild
Savur 063	*Cicer reticulatum*	Turkey	Wild	Wild
Sirna 060	*Cicer reticulatum*	Turkey	Wild	Wild

Table S.3.1 Statistical information for all measurements and groups.

Supplemental Table S1

	Wild						Domesticated						Treatment			History			Treatment * History		
	1 ppm			100 ppm			1 ppm			100 ppm											
Measurment	Sample Size (n)	Mean	SE	Sample Size (n)	Mean	SE	Sample Size (n)	Mean	SE	Sample Size (n)	Mean	SE	p-value	df	t-statistic	p-value	df	t-statistic	p-value	df	t-statistic
Carbon content (%)	99	38.00	1.06	116	41.37	0.22	40	38.36	0.34	41	42.64	0.37	**0.002**	296	3.09	0.763	294	-0.30	0.574	296	-0.56
Nitrogencontent (%)	99	1.02	0.06	116	2.38	0.09	40	1.23	0.05	41	2.61	0.19	**<.001**	277	7.85	0.232	56	-1.21	0.898	277	-0.13
Aboveground Biomass (g)	99	0.09	0.01	116	0.31	0.03	40	0.15	0.02	41	0.55	0.06	**<.001**	275	7.59	0.281	50	-1.09	**0.004**	275	-2.93
Average Root Diameter (mm)	99	0.47	0.01	116	0.57	0.02	40	0.54	0.02	41	0.70	0.07	**<.001**	277	3.35	0.138	52	-1.15	0.342	277	-0.95
Belovground Biomass (g)	99	0.23	0.01	116	0.50	0.03	40	0.34	0.03	41	0.66	0.08	**<.001**	274	5.10	0.075	43	-1.82	0.656	274	-0.45
Canopy Photosynthesis (µmol m^2 s)	20	346.77	98.04	21	3391.85	482.48	23	751.54	113.15	23	7968.61	1217.99	**<.001**	76	8.43	0.800	21	-0.26	**0.001**	76	-3.34
Carbon to Nitrogen Ratio (C/N)	99	39.05	0.63	116	19.94	0.70	40	32.90	1.10	41	20.09	1.49	**<.001**	276	-8.06	**<.001**	55	4.04	**<.001**	276	-3.39
Chlorophyll Content Index	99	16.19	0.61	116	34.75	1.20	40	18.92	1.27	41	36.28	1.83	**<.001**	274	8.10	0.204	45	-1.29	0.654	274	0.45
N investment in Chlorophyll	99	17.08	0.68	116	16.67	0.85	40	15.79	1.07	41	17.25	1.61	0.376	272	0.89	0.550	40	0.60	0.345	272	-0.95
Maximum Photosynthetic Rate per area (µmol m^2 s)	20	3.88	0.46	21	18.68	1.30	23	7.22	0.89	23	35.01	3.65	**0.005**	76	10.46	0.358	24	-0.94	**0.001**	76	-3.37
Maximum Photosynthetic Rate per mass (µmol g s)	20	2.15	0.24	21	9.41	0.54	23	2.90	0.29	23	13.39	1.19	**<.001**	76	11.96	0.559	21	-0.59	**0.013**	76	-2.54
PNUE per area (Aa/N)	20	2.25	0.25	21	3.97	0.35	23	2.41	0.27	23	5.99	1.09	**<.001**	77	4.46	0.911	28	-0.11	0.111	77	-1.61
PNUE per mass (Am/N)	20	3.94	0.48	21	7.56	0.74	23	6.02	0.83	23	15.74	3.12	**<.001**	77	4.27	0.463	31	-0.74	0.068	77	-1.85
Root Density (g/cm^3)	99	0.14	0.01	116	0.12	0.01	40	0.10	0.01	41	0.17	0.02	**<.001**	278	3.57	0.050	60	2.00	**<.001**	279	-3.85
Root Length (cm)	99	1031.80	39.55	116	1815.67	78.38	40	1352.46	91.62	41	2036.13	133.43	**<.001**	270	4.69	0.059	38	-1.94	0.537	270	0.62
Root Surface Area (cm^2)	99	157.62	7.73	116	362.67	19.26	40	248.43	21.70	41	493.09	49.45	**<.001**	272	6.21	0.056	38	-1.97	0.571	272	-0.57
Root Volume (cm^3)	99	1.77	0.11	116	5.23	0.47	40	3.39	0.46	41	10.05	2.41	**<.001**	276	4.57	0.250	51	-1.16	0.082	276	-1.75
Specific Leaf Area (cm^2/g)	20	177.47	6.55	21	199.45	7.70	23	241.43	7.82	23	255.16	8.59	0.193	87	1.31	**<.001**	87	-5.90	0.590	87	0.54
Specific Root Length (cm/g)	99	4911.78	139.29	116	4906.44	246.00	40	5340.07	209.08	41	3835.00	200.75	**<.001**	296	-3.49	0.239	296	-1.18	**0.003**	296	2.95
Stomatal Conductance (µmol m^2 s)	20	0.03	0.01	21	0.14	0.02	23	0.06	0.01	23	0.38	0.11	**<.001**	296	4.41	0.792	24	-0.27	**0.045**	76	-2.03
Water-use Efficiency (δ13C)	99	-31.60	0.07	116	-31.47	0.08	40	-32.12	0.12	41	-31.25	0.14	**<.001**	272	5.64	**0.018**	38	2.48	**<.001**	273	-4.11

3.9 Figures

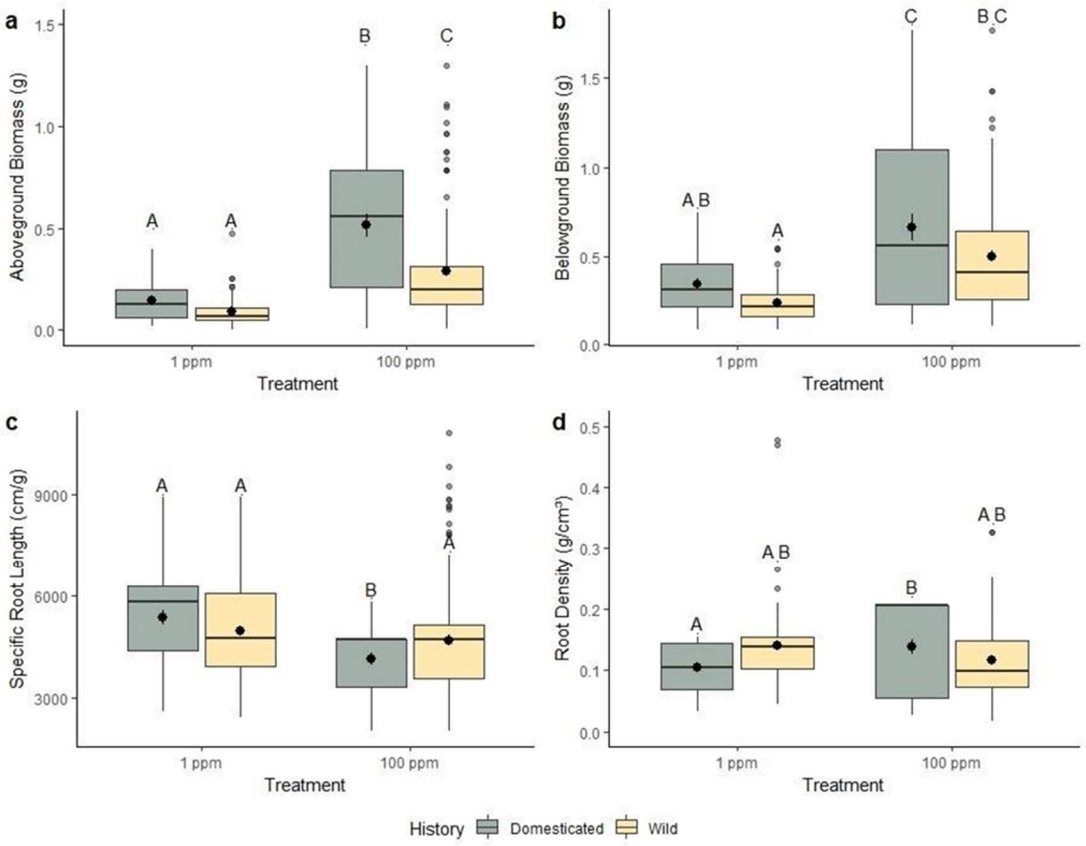

FIG. 3.1 The response of chickpea morphology to increased nitrogen availability. (a) Aboveground Biomass, (b) Belowground Biomass, (c) Specific Root Length (SRL), and (d) Root Density. Domesticated (yellow) and wild (green) chickpea accessions are grouped. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's HSD test).

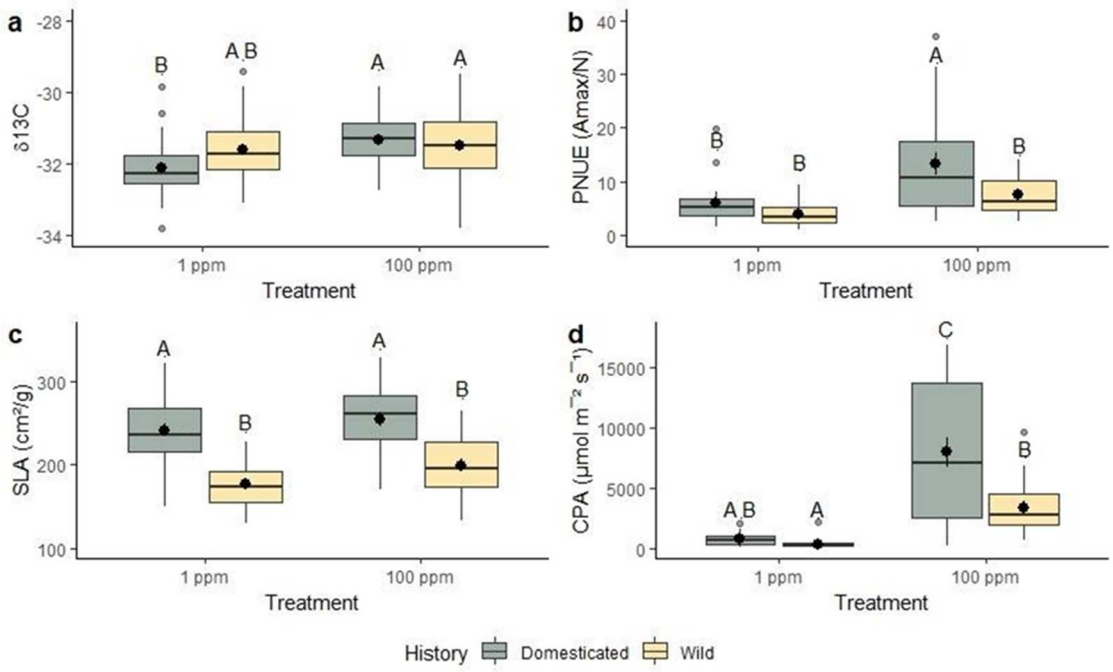

FIG. 3.2 The response of chickpea morphology to increased nitrogen availability. (a) Water-use Efficiency (δ13C), (b) Photosynthetic Nitrogen-use Efficiency (PNUE), (c) Specific Leaf Area (SLA), and (d) Canopy Photosynthesis (CPA). Domesticated (yellow) and wild (green) chickpea accessions are grouped. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's HSD test).

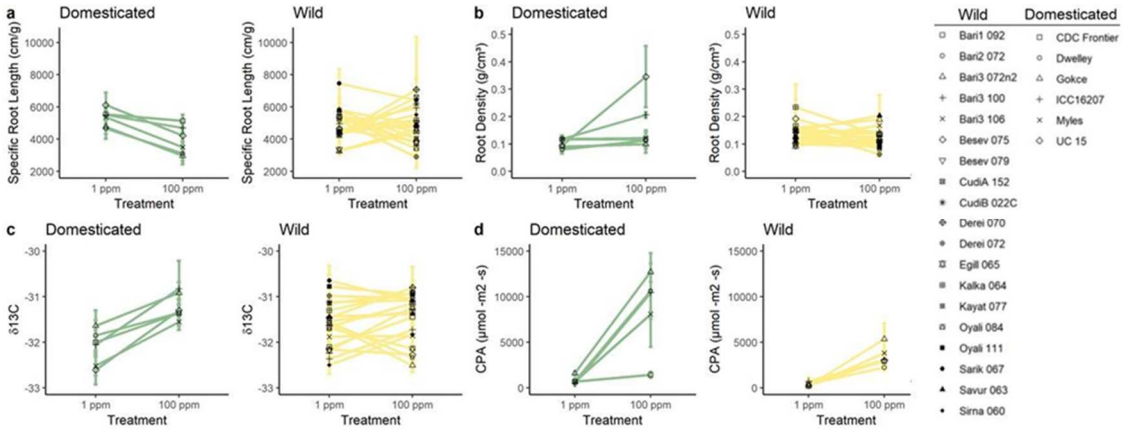

FIG. 3.3 Trait means of individual chickpea accessions in low (1 ppm) and high nitrogen environments(100 ppm). (a) Specific Root Length (SRL), (b) Root Density (c) Water-use Efficiency (δ13C), and (d) Canopy Photosynthesis (CPA). Domesticated (yellow) and wild (green) chickpea accessions are grouped. Error bars denote standard errors ±.

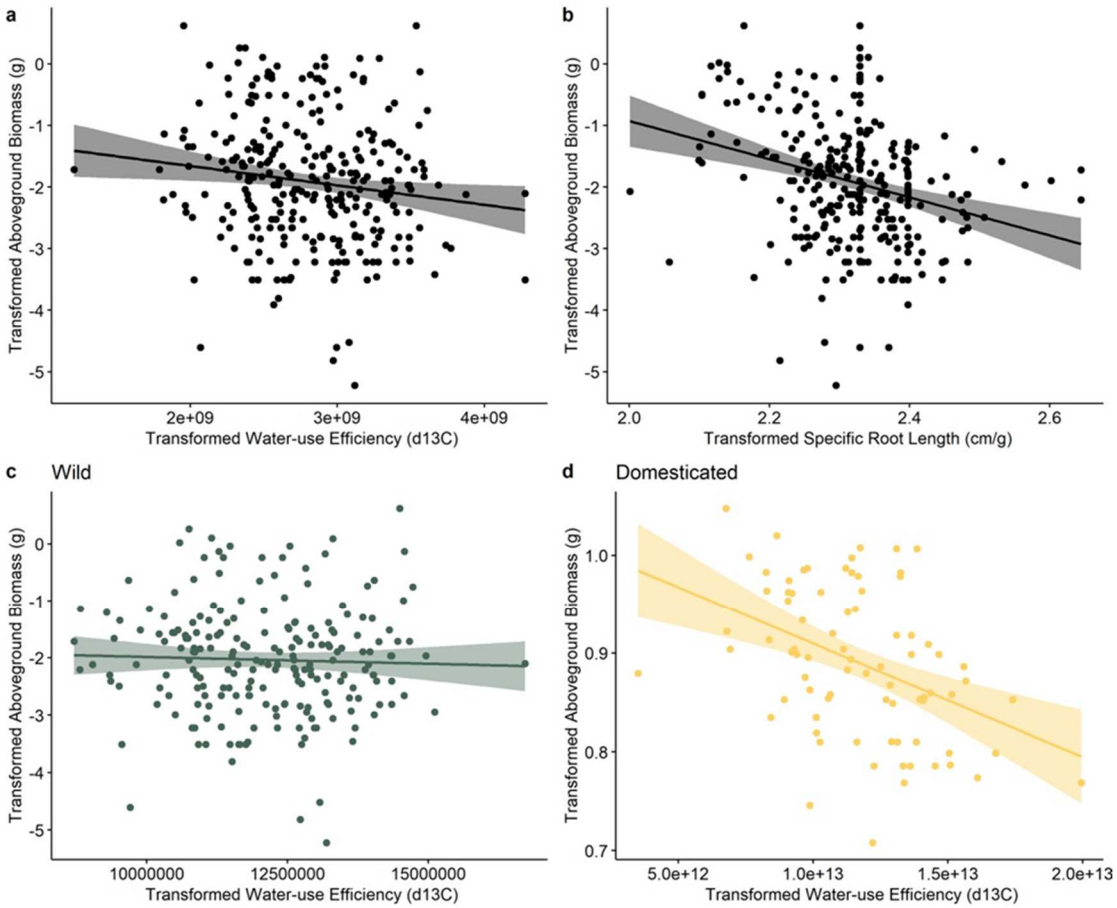

FIG. S3.1 Supplemental Material S1. Correlation of plant plasticity to plant fitness. (a) Transformed aboveground biomass vs. transformed water-use efficiency (b) Transformed aboveground biomass vs. Transformed specific root length (c) Transformed aboveground biomass vs. transformed water-use efficiency for wild chickpea (d) Transformed aboveground biomass vs. transformed water-use efficiency for domesticated chickpea. Black line represents best fit. Yellow dots denotes domesticated chickpea data points, green dots denotes wild chickpea data points, and black dots denote wild or domesticated chickpea data points. Shaded regions represent 95 % confidence intervals.

CHAPTER 4: DEFINING AND IMPROVING THE "ROTATIONAL" AND "INTERCROPPING VALUE" OF A CROP USING A PLANT-SOIL FEEDBACKS APPROACH

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4.2 Abstract

Crop rotations and intercropping are an ever-present sustainable practice across a diverse array of agroecosystems. These management practices can suppress weeds, reduce cycles of disease, build soil organic matter, and increase above and below ground biodiversity, all of which improve the yield of a companion or subsequent crop. Here, we propose the terms “rotational” and “intercropping- value” as a way to measure the overall effect of these benefits. Additionally, we articulate how to quantify different ecosystem services provided by rotational and inter-crops, including weed and disease suppression, enhancing microbial communities, and nitrogen fixation. By providing a way of identifying and quantifying these rotational and intercropping traits, it might provide an ideotype to facilitate the breeding of better crops for rotations, for cover cropping, and for intercropping.

4.3 Introduction

Temporal and spatial alternation of crop species is a very common practice of most annual and short duration agricultural systems. Rotations and intercropping of crops can break cycles of disease and pests, improve soil fertility, suppress weeds, and improve food and nutritional security (Reviewed in Fageria et al., 2005; Sharma et al., 2018; Wick et al., 2017). Although rotations and intercrops are widely used in agriculture, the effect of a crop as a rotational or intercrop partner is not typically estimated or systematically studied (Ingerslew & Kaplan, 2018), let alone considered as a breeding target. In a recent meta-analysis of 154 studies, it was discovered there were no science-based criteria to justify the use of one crop rotation or another (Dias et al., 2015). Here we coin the terms "rotational value" and "intercropping value" and provide an equation to measure the utility of crops in spatial and temporal mixtures. By precisely measuring and quantifying the value of cover crops and intercrops, we can provide scientists, farmers, and policy-makers with estimates of value that can encourage more sustainable rotations that may provide greater long-term agricultural and ecological benefits.

4.3.1 *Plant-Soil Feedbacks: A Potential Framework to Quantify Rotational and Intercropping Value*

A method to quantify rotational and intercropping value is to use the framework of plant-soil feedbacks (PSF, e.g., Bever 1994, 2003; Callaway et al., 2004; Ehrenfeld et al., 2005; Kulmatiski et al., 2008; van der Putten et al., 2016; van Nuland et al., 2016), a powerful approach for interpreting the effects of one species of plant on other species indirectly through their impact on soil biota or soil chemistry (Reviewed in Mariotte et al., 2018). Although the majority of PSF research has primarily focused on natural systems,

the temporal and spatial crop diversification within agricultural systems provide an excellent model to utilize the PSF framework to increase agriculture production (Barel et al., 2018; Cheng and Cheng, 2015; Huang et al., 2013; Mariotte et al., 2018; Wang et al., 2017). Thus, we can improve agricultural production by measuring the direction and strength of PSF for different crops or management practices, and implementing the practices with the highest PSF values (Barel et al. 2018; Huang et al., 2013; Ingerslew & Kaplan, 2018; Mariotte et al., 2018). The concept of PSF has underutilized potential in agroecosystems, therefore, we adapted this concept and coined the terms rotational value (*RV*) and intercropping value (*IV*) and defined the terms as to how well a crop relatively benefits the yield or growth of another crop. We believe the terms rotational and intercropping value directly conveys a non-specialist meaning of the PSF concept that can be easily understood by the entire agricultural community. Additionally, we provide an equation to measure rotational value (*RV*) and intercropping value (*IV*) adapted from PSF (Bever et al., 1994; Ingerslew & Kaplan, 2018; Wang et al., 2017):

RV or $IV = \ln(Y\varepsilon/Yc)$

$Y\varepsilon$ = yield or growth of the subsequent crop on rotated soil or grown with an intercrop.

Yc = yield or growth of the subsequent crop on control soil (non-rotated soil or grown with no intercrop)

0 = no rotational value or intercropping value (has no effect on yields or growth compared to control soil)

0 < = positive rotation value or intercropping value (increases yields or growth compared to control soil)

0 > = negative rotational value or intercropping value (decreases yields or growth compared to control soil)

The benefit of using PSF to measure rotational and intercropping value is that it provides a straightforward framework to consistently quantify the effect of a cover crop or intercrop on the yield or growth of another crop. Additionally, by calculating a numerical value that normalizes results by comparing them to non-primed soil (non-rotated soil or no intercrops present), it provides scientists, breeders, and farmers with a simple quantifiable measurement to compare agricultural practices and crops using "classical" statistics. Furthermore, when comparing rotational and intercropping values among crops or management practices, we strongly suggest that soil chemistry and soil history (previous planted crops, management practices, pest presence, etc.) should be used as a covariate to control for differences between field sites, to alleviate potential drawbacks and increase the applicability of results. For instance, research has shown that the legacy of land-use history can influence plant physiology; consequently, the results obtained in cover crop and intercrop studies may be in part due to a legacy effect (Li et al., 2019).

Although the utilization of the PSF framework to quantify the rotational value or intercropping value is straightforward and can be easily conducted in a field or greenhouse setting, this approach may overlook a broader range of long term agroecological benefits. For example, relatively short term rotational experiments of a couple of seasons may overlook longer-term benefits, such as increased soil contribution, microbial activity, or a

broader set of ecosystem services, that are more likely to be apparent over longer rotational cycles or geographic scales (Capó-Bauçà et al., 2019; Gabriel et al., 2016; Schmidt et al., 2018). However, long term experiments are harder to replicate on a wide scale, and often exceed the timeframe on which researchers need to complete research.

Furthermore, this approach does not estimate or identify the underlying mechanisms that contribute to the rotational or intercropping value. Understanding the underlying mechanisms that influence rotational and intercropping value provides scientists with predictive insight into why certain crops and management practices are beneficial, while also allowing the identification of potential breeding targets. For instance, if the rotational value of legumes is primarily derived by its ability to host symbiotic nitrogen-fixing rhizobia, the PSF framework would not be able to identify these traits as the primary mechanism contributing to its rotational value. These mechanisms would only be identified by measuring nitrogen fixation among all treatment groups during the experiment. Similarly, the rotational value of a daikon-type tillage radish is primarily derived by its ability to act as a biodrill. The daikon-type tillage radish has the capacity to penetrate a compacted soil, as well as "mop-up" excess nutrients left in a field by a previous crop (e.g., Chen and Weil, 2010; Gruver et al., 2014). These mechanisms would only be identified by measuring soil compaction, as well as other traits such as root architecture and radish breakdown rate (Gruver et al., 2014). Thus, to gain a more comprehensive understanding of the effects of management practices in agroecosystems and to identify potential breeding targets, we believe that rotational and intercropping studies need to measure ecosystem services in addition to calculating the RV and IV of crops and management practices. The literature on ecosystem services and their valuation is too vast

and complex to review here (readers are directed to de Groot et al., 2002; Guerry et al., 2015; Power, 2010; Swinton et al., 2007; Zhang et al., 2007, for a small sample of the growing literature), but our framework can easily handle any ecosystem service.

4.3.2 *Ecosystem services could be bred for to improve rotational and intercropping values*

Crop rotations and intercropping are an aspect of many contemporary and historical agricultural systems that provide several short- and long-term benefits (Reviewed in Fageria et al., 2005; Sharma et al., 2018; Wick et al., 2017). Rotations and intercrops, in general, provide many ecosystem services such as suppressing weeds, hindering disease cycles and pest outbreaks, sequestering carbon to build soil organic matter, supporting pollinator and natural enemy populations, and helping mobilize other limiting nutrients, such as phosphorus (Altieri et al., 1984; Krupinsky et al., 2002; Teasdale et al., 2004; Snapp et al., 2005; Wick et al., 2017). These diverse rotational and intercropping ecosystem services can be quantified in numerous ways (see above) and, thus, can be treated as rotational and intercropping traits that can be selected and bred for to enhance rotational and intercropping values (Figure 1). For instance, if we continue with the previous example of legumes, we could increase the rotational value of legumes, by breeding legumes for enhanced nitrogen fixation, whether through increased nodulation or a broader range of host specificity with rhizobia. Alternatively, in the case of cereals, we can potentially increase their rotational value by increasing their ability as weed suppressors or nutrient scavengers, by breeding for enhanced ground cover, allelopathy, or nutrient acquisition (e.g., Worthington and Reberg-Horton, 2013). Therefore improving the ecosystem services that a cover crop or intercrop provides should hypothetically increase their rotational or

intercropping value. However, breeding for the enhancement of an ecosystem service can be challenging because determining the most appropriate measurement for each service can be difficult. Most ecosystem services are not as simply quantified as other agronomical traits such as plant height, yield, and tolerance to biotic or abiotic stress (e.g., de Groot et al., 2002; Zhang et al., 2007). We explore this dilemma in the upcoming paragraphs for key rotational and intercropping traits and identify different methods of quantifying ecosystem services that may be useful for the enhancement of rotational and intercropping values (e.g., Schipanski et al., 2014).

A commonly utilized rotational and intercropping trait of crops is the suppression of weeds. Within crops, legumes may perform more poorly than cereals and may permit greater weed biomass (Baraibar et al. 2018; Hodgdon et al., 2016). Thus, mixtures of different crops may be most effective at weed prevention (Baraibar et al., 2018; Florence et al., 2019). Crop mixtures that are sown as polycultures, or undersowing a primary crop with a shorter stature secondary crop, provide a greater competitive impact on weeds, thereby raising yields (Chauhan et al., 2012). Weed suppression can be measured in weed control savings, weed abundance, or weed biomass. The risk, as with other rotational and intercropping traits, is that weed pressure is notoriously variable, so estimates are context-dependent. In addition to the quantifying weed suppression, identifying the mechanisms of how the crop is suppressing weeds (shading, allopathy, or nutrient acquisition) is imperative for the breeding process; as it will narrow the range of traits that breeders or farmers will need to select for to increase rotational or intercropping values (Florence et al., 2019; Worthington and Reberg-Horton, 2013). Furthermore, the utilization of highly

diverse multi-species mixtures to reduce weeds may lead to a refinement of traits that are compatible with mixtures.

Another important aboveground rotational and intercropping trait is the increase of aboveground biodiversity and the suppression of pests (Smith and McSorely, 2000). Rotational and inter-crops have been shown to increase the presence of pollinators and natural predators of pests; an ecosystem service hypothesized to reduce yield gaps in agroecosystems (Bommarco et al., 2012; Hummel et al., 2002; Rusch et al., 2013). For instance, cover crops and intercrops were seen to increase the presence of pest predators in cotton (Tillman et al., 2004) and broccoli (Ponti et al., 2007). Additionally, ground cover in almond orchards was correlated with increased pollinator presence, specifically increasing native bee presence (Saunders et al., 2013). Due to the direct correlation this trait has on yield, aboveground biodiversity is a key contributor to a crops' rotational and intercropping value and should be measured. This trait can be measured by numerous methods such as abundance and/or presence of pests and pollinators, and herbivory damage (Buckland et al., 2005). However, similarly to weed suppression, identifying the mechanisms as to why certain crops increase aboveground biodiversity or deter pests is imperative to the breeding process. For instance, some crops may give off volatile compounds, while others provide resources to attract pollinators (e.g., Baldwin, 2010) or natural enemies like ants (e.g., Jones et al., 2016). Identifying which plant mechanisms helps provide the ecosystem service will supply breeders and farmers with a narrowed selectable trait list to increase rotational and intercropping values.

A key contributor to rotational and intercropping value is the contribution plants make to the soil. Plants contribute to the soil in two primary ways: through exudates, mixtures of organic compounds from their roots that can constitute a staggering 20-40% of the entire metabolism of a plant, and as decaying roots and above-ground parts that remain in the soil or are incorporated into the soil after the plant senesces (e.g., Kuzyakov and Domanski, 2000). Root exudates likely are involved in several key functions (Friesen et al., 2011, van Dam and Bouwmeester 2016): 1. providing carbohydrates to beneficial symbiotic soil partners like rhizobia and mycorrhizal fungi; 2. recruiting other growth-promoting or "defensive" microbes to the root surface; 3. helping plants obtain limiting nutrients such as phosphorus and iron that adhere strongly to soil particles and can be released when roots secrete weak organic acids to dissolve them; 4. buffering the effects of potentially toxic aluminum and heavy metals in the soil; 5. inhibiting pathogens and herbivores such as bacteria, fungi, nematodes, and soil insects. Although these functions are well known, measuring exudate variation across soil conditions, between species and genotypes, or as interactions between genotype and environment is quite challenging because soil microbes will metabolize root exudates upon their release from roots (e.g., Jacoby and Kopriva 2018; Oburger and Jones, 2018; Petriacq et al., 2017; van Dam and Bouwmeester, 2016).

Despite the limitations of quantifying root exudation, one can quantify the effects of rotations and intercrops on microbial soil communities. Understanding the effect of crops on microbial diversity and functional activity are critical since they have been positively correlated with soil health and crop productivity (McDaniel et al., 2014; Tiemann et al., 2015). For instance, it has been shown that cover crops increased microbial

diversity and/or activity in agroecosystems which led to increased yields in potatoes, *Solanum tuberosum* (Larkin et al., 2010), grapes, *Vitis* (Ingels et al., 2005), cucumbers, *Cucumis sativus* (Tian et al., 2011) and cotton, *Gossypium hirsutum* (Mbuthia et al., 2015; Nouri et al., 2019). Additionally, in the past two decades, methods for measuring soil microbial communities have changed radically, opening up new possibilities for research on plant-microbe interactions. These new methods to quantify soil microbial communities fall into three community characterization categories: size, composition, and activity (Reviewed in Harris, 2006). There are benefits and limitations to each measurement and characterization approach, and deciding which approach to utilize is dependent on how microbial communities increase rotational and intercropping values. For example, if the rotational or intercropping value is increased with an increase in soil microbial populations, then characterizing the microbial community by its size through microbial biomass measurements is sufficient. However, if a specific microbial community composition increases rotational or intercropping value, then a more precise and advanced shotgun metagenomics approach will be necessary. Nevertheless, all characterization approaches are quantifiable and, therefore, should be measures we can integrate into breeding programs.

Furthermore, one can calculate the ability of a crop to break cycles of disease attack by the decreased cost of pesticides or by infection rates (Larkin et al., 2010). With next-generation sequencing, one can measure pathogen presence and population size before and after a rotation and compare different rotations and their impact on pathogen presence, making a much more precise measure of this benefit. Furthermore, a rotational or inter-crop may help recruit antagonists of pathogens, such as *Trichoderma* and *Pseudomonas*

which induces plant immune responses (Bakker et al., 2007; Han, 2019; Korolev et al., 2008; Shoresh et al., 2005; Vitti et al., 2016). For instance, Wang et al. (2017) found positive legacy effects of intercrops believed to be due to shifts in soil communities, which reduced the negative effects of soil pathogen build-up. However, identifying these mechanisms of disease suppression requires experimental designs that can take several seasons to implement. Moreover, these trait measurements estimated from experiments, particularly at a single site, are highly context-dependent and may depend on the presence of natural predators or other local factors impacting the presence of pathogens. Understanding this context-dependence can allow one to manipulate natural predators encouraging their populations or making them available in particular rotations (e.g., Jones et al., 2016). Altogether, this makes breeding for disease suppression a complex task.

Lastly, for legumes, an important rotational and intercropping trait is nitrogen fixation. However, legume hosted biological nitrogen fixation does vary substantially depending on the species, the genotype, the availability of efficient symbiotic rhizobia in a particular field setting, and the effectiveness of the symbiosis in that field setting (Busby et al., 2017; Hardarson et al. 1993; Vyn et al., 2000). Nitrogen fixation tends to be lower in field settings compared to laboratory settings due to factors such as poor adaptation to the host, poor adaptation of the rhizobia to the soil, abiotic stress that limits the effectiveness of the symbiosis, or other factors (see Busby et al., 2017; Thrall et al., 2009). However, breeding for enhanced nitrogen fixation overlooks the timing of nitrogen availability. Depending on management conditions, only some nitrogen from a subsequent cover or rotational crop is likely to be available to the next crop (Burity et al., 1989; Vyn

et al., 2000). As a result, this will most likely affect true rotational and intercropping values in a field setting.

4.3.3 *Developing ideotypes for rotational and intercropping value: Next-generation breeding targets*

All of these conceptions of rotational and intercropping traits that increase the value of rotational and inter-crops expand the range of breeding targets that can be used in developing more effective crop rotations and intercrops. Using ideotypes to breed for particular crop phenotypes remains a powerful conceptual framework (Donald, 1968). Our definition of rotational and intercropping value fits well into the ideotype breeding approach. For instance, if a legume is the breeding target, association with a broader range of rhizobia may be a beneficial trait to select for to increase nitrogen fixation, which in turn increases its rotational and intercrop value. For tillage crops, such as daikon radishes, this may be the extent of soil compaction and timing of nutrient uptake and release. For cereals used as covers, it may be growth rate, allelopathy, or cold hardiness. Across all crops used for rotational benefits or intercropping, breeders will ideally have a range of traits that can be measured effectively, such as increasing soil organic matter (with favorable C:N ratios), providing weed suppression, lowering infection rates of diseases, and mobilizing nutrients. These are all traits for which genetic variation in crops almost certainly exists, and could be useful breeding targets to increase agricultural sustainability and productivity.

4.4 References

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4.9 Figures

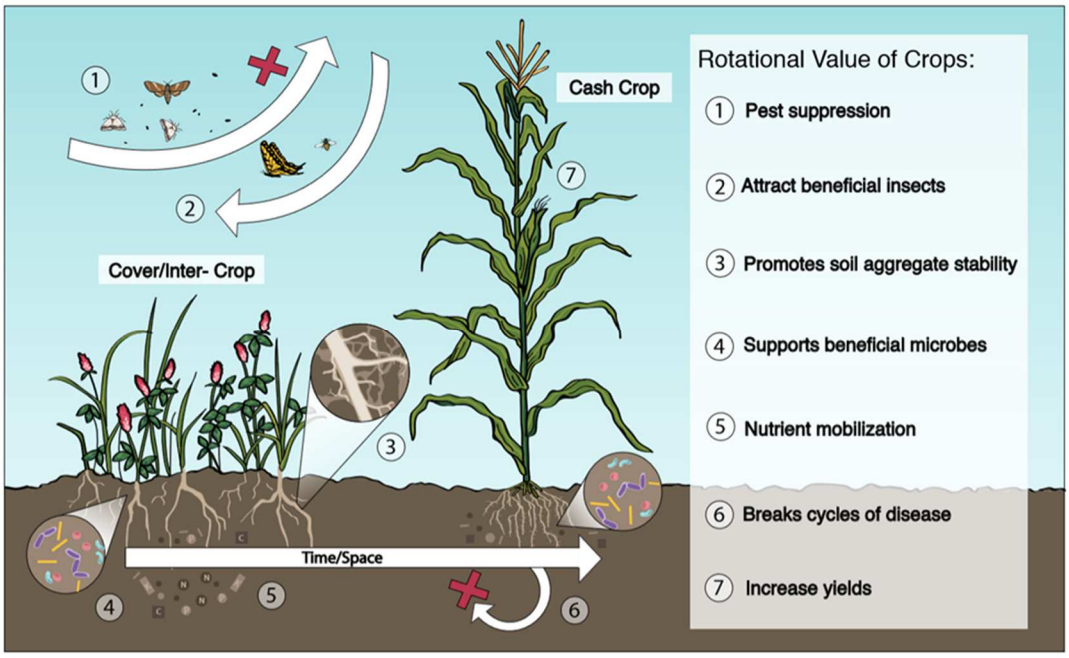

FIG. 4.1 Temporal (e.g., crop rotation) or spatial (e.g., intercropping) crop biodiversity can benefit cash crops in numerous ways such as: 1. suppressing pests, 2. attracting beneficial insects, 3. promoting soil aggregate stability, 4. supporting beneficial microbes, 5.mobilizing nutrients, and 6. breaking cycles of diseases. These benefits can contribute to the rotational or intercrop value of a crop, or how well a crop benefits the yield or growth of a cash crop. By identifying which benefit(s) facilitates the yield or growth of a crop, we can select for the enhancement of that trait(s), thus increasing the rotational or intercrop value of the crop and making them a better rotational or intercropping partner.

CHAPTER 5: FIELD PEA A NEXT-GENERATION COVER CROP; INDENTIFYING INTRASPECIES VARIATION FOR COVER CROPPING TRAITS

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5.1 Abstract

To sustainably meet the caloric demands of the growing human population, we must decrease the yield gap between sustainable and conventional agricultural practices. A possible solution to mitigate this yield gap is through the improvement of cover crops as rotational partners. However, to improve cover crops as rotational partners, intraspecies variation for cover cropping traits such as nutrient mobilization, carbon deposition, and beneficial microbial recruitment must be identified. Predominately cover crop research has focused on interspecies comparisons for cover cropping variation with minimal research investigating intraspecies variation. Therefore, to address if variation of cover cropping traits is present within a cover cropping species, we grew 15 accessions (four modern cultivars, three landraces, eight wild accessions) of field pea in an organic setting We measured various cover cropping traits such as nutrient mobilization, soil organic matter deposition, microbial recruitment, and quantified the effect of the field pea accession on the growth and yield of a subsequently planted corn crop. We found that domestication history and genotype of field pea had a significant effect on soil properties: C%, N%, manganese, magnesium, sodium, calcium, and effective CEC, and the yield of the subsequent corn crop. Additionally, no variation for microbial recruitment was observed within field pea, but when compared to control soil, peas as a whole were enriched with growth-promoting bacteria *Firmicutes* and *Patescibacteria*. In conclusion, our results revealed the presence of intraspecies variation for cover cropping traits which, may have impacted the rotational values of field pea accessions. In summary, our results demonstrate

that cover crops can be improved as rotational partners to ultimately boost crop yields in sustainable agroecosystems.

5.2 Introduction

Cover crops are ubiquitous in agroecosystems due to their beneficial impacts on crop yields, above and belowground biodiversity, disease and weed suppression, and overall soil health (reviewed in Hartwig & Ammon, 2002; Sharma et al., 2018; Snapp et al., 2015; Miguez and Bollero, 2005). Due to these benefits, the number of agricultural acres being cover cropped in the United States has increased by 49.7% from 2012 to 2017 (USDA, 2019). Despite the popularity of cover crops and their primary role in sustainable agriculture, minimal effort has gone into improving cover crops as rotational partners. The majority of cover crop research has been focused on comparing cover cropping traits between species or species mixtures, whereas few studies have investigated differences between cover cropping traits within species. Identifying intraspecies differences may provide the foundation for improving the rotational value of cover crops, which we define as a measure of how well a cover crop increases the yield of a subsequent crop (Marques et al., in review). To this end, increasing rotational value may potentially offset the estimated 19.2% yield gap between conventional and sustainable agricultural practices (Ponsio et al., 2015; Sharma et al., 2018). Reducing the yield gap between these systems through rotational value improvement is critical for making sustainable agriculture practices a feasible solution to feed the ever-growing human population (Muller et al., 2012; Licker et al., 2010; Ponsio et al., 2015; Barbieri et al., 2019; Sharma et al., 2018).

Differences in cover cropping traits among species and families including weed and disease suppression (Snapp et al., 2005), soil organic matter deposition (Johanning, 2014), nutrient mobilization (Hallama et al., 2019), and below (Liang et al., 2014; Wagg et al., 2011) and aboveground (Finney and Kaye, 2017) biodiversity improvement, have been well documented in various agroecosystems (reviewed in Hartwig & Ammon, 2002; Sharma et al., 2018). For instance, when compared to cereal cover crops, legume cover crops are not well suited for weed suppression (Hodgdon, Warren, Smith, & Sideman, 2016; Chauhan et al., 2012) but are more efficient at increasing soil carbon and nitrogen (Austin et al., in review; Snapp et al., 2005). Despite these well-established cover crop generalizations, most cover cropping studies are limited in that they use a single variety or accession to represent an entire cover cropping species. Thus, the use of a single accession or variety is problematic because within-species variation for agronomically important traits, such as abiotic tolerance (reviewed in Rao et al., 2016; (Zhang et al., 2016; Bosetti et al., 2011; reviewed in Bita & Gerats et al., 2013; Govindaraj et al., 2018), and resistance to diseases (Vasudevan et al., 2014; Silvar et al., 2010) and pests (Rakha et al., 2017; reviewed in Broekgaarden et al., 2011), have been consistently found across crops. As a result, intraspecies variation for cover cropping traits such as nutrient mobilization, organic matter deposition, and beneficial soil microbial recruitment most likely exist. Therefore, cover cropping results from a single variety or accession must be carefully extrapolated since it may lead to incorrect generalizations about crop families or species.

In addition to increasing the number of accessions and varieties used in studies, the incorporation of crop wild relatives (CWRs) in cover cropping research should be considered. Tribououillois et *al*. (2015) suggested that domestication has reduced adaptive

strategies and modified leaf trait syndromes in cover crops. Thus, the impacts of genetic bottlenecks associated with domestication and modern breeding (Doebley, Gaut, & Smith, 2006) may also be affecting the genetic and phenotypic diversity of cover crops. To alleviate restrictions on genotypic and phenotypic diversity, the incorporation of genetic material from CWRs that have not undergone domestication may help increase intraspecies variation in cover cropping studies.

Here we test if rotational traits and values vary within cover cropping species by utilizing a modified plant-soil feedback (PSF) framework and an assortment of pea accessions with varying domestication histories (modern cultivars, landraces, and wild peas,). We measured various cover cropping traits such as nutrient mobilization, organic matter deposition, microbial recruitment, and rotational values. We hypothesized that cover cropping traits will vary between pea accessions and domestication histories, as other agronomically important traits have been seen to vary between pea accessions (Smykal et al., under review). The presence of variation in cover cropping traits would suggest that rotational values in cover crops can be enhanced to potentially increase yields in agroecosystems.

Field pea was selected because it is the second-highest planted legume cover crop and is utilized as a spring and winter cover crop throughout the United States (SARE, 2017). Field pea is a popular cover crop because of its numerous benefits: it can fix 90–150 pounds of nitrogen per acre; it is transpiration efficient (i.e., it converts a small amount of water into a large amount of biomass); and it provides disease suppression (SARE, 2007;

USDA, 2016). Additionally, it can be terminated organically and breakdowns quickly, making it an ideal green manure for all agroecosystems (SARE, 2007).

5.3 Materials and Methods

5.3.1 Plant Material

Fifteen field pea accessions were used in this experiment. All accessions were requested from the USDA-NPGS and then amplified in Burlington, Vermont. Eight of the accessions, W6 26154, W6 26154 PSP, W6 26157, W6 26157 PSP, W6 26159, W6 26160 PSP, W6 26161, and W6 26161 PSP, were wild accessions from the country of Georgia. The remaining accessions, PI 269761, PI 269761 PSP, PI 639977 PSP, and PI 639981 PSP, were modern cultivars originating from the Czech Republic (2) and Bulgaria (2) respectively, and PI 577142, W6 3674, and W6 3675 were landraces from Nepal. The use of plant material from different geographic origins and domestication history made it possible to capture a wide range of genetic and phenotypic diversity.

5.3.2 Experimental Design

Four replicates of each pea accession and two controls (no cover crop and no cover crop with fertilizer) were grown or administered in a randomized block design in an organic field at the University of Vermont Horticulture Research Center in South Burlington, Vermont. Approximately 20.41 g of each field pea accession was planted at a depth of ~2.54 cm in 2.8m² plots. The sowing rate of ~7.3 g/m² and the 2.54 cm planting depth mimicked the recommended cover cropping plant density of 65 lbs/ac for cover cropping field peas (NDSU, 2002; Stepanovic, 2017). Before planting, all seeds were sterilized with a 1% bleach solution to ensure no microbes were introduced to the plot via the seed coat.

Plots were irrigated as needed. Pea plants were grown for 44 days, after which soil rhizosphere samples were collected, and the total number of plants in the plot, the average plant height, and the average aboveground biomass were recorded. To calculate the average plant height, three of the most center plants in the plot were selected, and plant height (base of the plant at soil level to the top of the stem) was recorded and averaged. After their height was measured, the plants were uprooted, and soil rhizosphere samples were collected. The rhizosphere was defined as any soil still clinging to the root of the plant after the plant was uprooted. For control plots, bulk soil was taken at an approximate depth of 15 cm. To calculate the average aboveground biomass, the three uprooted plants' aboveground portions were separated from their belowground portions and oven-dried for 48 hours at 49□ and then weighed using an analytical scale. After pea plant measurements were recorded, soil core samples were collected at the center of each plot at an approximate depth of 15 cm using a 7.5 cm diameter soil recovery AMS auger. Soil samples and the previously listed plant measurements were obtained from the plots' centers to avoid edge and interacting effects from neighboring plots. Soil core samples were then sent to the University of Vermont Agricultural and Environmental Testing Laboratory, where they were tested for pH, % nitrogen (N), % carbon (C), % soil organic matter, phosphorus, potassium, aluminum, calcium, copper, iron, magnesium, manganese, sulfur, zinc, and effective cation exchange capacity (CEC). After the soil core samples were obtained, the remaining plants in the plots were hand-harvested by cutting the stem of the plant at soil level; this was done to minimize soil disturbance in the plot.

After harvesting pea plants, the sweet corn organic variety "Enchanted" was hand planted in the plots according to the manufacturer's specifications. "Enchanted" was used

because it is a neonicotinoid-free and late-season maturing variety that reaches maturity 78 days after sowing. Fertilizer was added to the "no cover crop with fertilizer" control plots according to the recommendations from the University of Vermont soil testing laboratory. However, this treatment was abandoned after a single application of fertilizer due to the mobility of nitrogen. Eighty days after sowing, the number of corn plants, average plant height, average aboveground biomass (cob and vegetative), and relative chlorophyll content was recorded for each plot (explained in more detail below). The same protocol that was used to calculate the average plant height and aboveground biomass of the pea plants was used for the corn plants. If a cob showed signs of pest damage, the measurement for that plant was excluded. For chlorophyll measurements, the youngest fully developed leaf and the oldest leaf were measured for leaf chlorophyll content using a Leaf Photosynthesis MultispeQ V1.0 (East Lansing, MI). Only plants closest to the direct center of the plot were sampled to avoid edge and interacting effects from neighboring plots.

5.3.3 Microbiome Measurements

Microbial DNA was extracted from bulk soil control plots and field pea rhizosphere samples using QIAGEN DNeasy PowerSoil Kits. Before DNA extraction, all samples were treated with propidium monoazide (PMA) using the manufacturer's protocol (Biotium) to prevent the extraction and amplification of soil relic DNA, which can potentially skew microbial diversity estimates (Carini et al., 2016). After extraction, DNA samples were sent to LC Sciences for DNA library preparation and 16S rRNA (V3 and V4 regions) sequencing using a next-generation MiSeq sequencer. Only one rhizosphere sample from each plot was sequenced. Sequence data was then processed for amplicon sequence variants (ASVs) using the requisite quality assurances in the Qiime2 and Dada2 pipelines

(Callahan et al., 2017). The taxonomy of the ASVs was characterized using the Ribosomal Database Project (RDP version 11.3), NCBI 16S Microbial Database, and the Greengenes databases.

5.3.4 Statistical Analysis

To calculate the effect of pea accessions on soil chemistry and the growth of the subsequently planted corn, a modified PSF framework was used, and the magnitude of PSF in each accession was calculated for all measurements (Marques et al., in review; Ingerslew & Kaplan, 2018; Mariotte et al., 2018). For all measurements, the following formula was used:

$$PSF_s = \ln\left(\frac{SM_s}{SM_c}\right)$$

Where SM_s is the recorded soil measurement or corn measurement of the plot, and SM_c is the average soil or corn measurement for all control plots. Additionally, to calculate the rotational value (RV), the same metric was used, where SM_s is the average corn cob weight of the plot, and SM_c is the average corn measurement of all control plots. The use of a standardized PSF and RV provides a clear understanding of the effect pea accessions have on soil chemistry and the subsequently planted crop. If PSF_s or RV was less than 0, then soil or corn measurements were lower than control measurements. If PSF_s or RV was greater than 0, then soil or corn measurements were higher than control measurements. Lastly, if PSF or RV was equal to 0, then soil or corn measurements were similar to control measurements.

A generalized linear mixed model was used to test for significant differences among accessions and histories (modern cultivar, landrace, wild) effects on soil chemistry and corn growth and yield (Bates et al., 2014). For soil measurement GLM models, block was used as a random variable, and the total aboveground biomass of the plot was used as a covariate. Total aboveground biomass of the plot was calculated by multiplying the number of pea plants in the plot by the average pea aboveground biomass of the plot. Although not a precise measure, this proxy gave an approximate estimate of the total aboveground biomass of the plot. This covariate was used to account for differences in pea plant size between accessions. For corn measurement GLM models, block was again used as a random variable, and the number of corn plants in the plot was used as a covariate. This covariate was used to account for differences in the number of corn plants present in each plot. A Tukey's HSD post-hoc test was used to test for significant differences between accessions and history groups. The effects of accession and history (domesticated or wild) on soil and corn measurements were analyzed separately, as the researchers wanted to test for significant differences between accessions. If both factors were included in a single model, accession would become nested within history and be categorized as a random term, thus preventing the identification of significant differences between accessions.

Additionally, to test for linear correlation between pea aboveground biomass of the plot and soil measurements, the Pearson correlation coefficient was calculated for all PSF soil calculations versus pea aboveground biomass. To test for linear correlation between rotational value and PSF soil calculations and pea measurements, the Pearson correlation coefficient was calculated for rotational values versus pea measurements or PSF soil calculations. All Pearson correlation coefficients were calculated using R's "psych"

package. A Levene's test was conducted for all measurements to test for equality of variances between accessions and history groups. All statistical analyses were performed in R (www.r-project.org).

5.4.1 Microbial Analysis

Amplicon sequence variants were used to calculate alpha diversity for both history and accessions using richness, evenness, Fisher's alpha index, and Simpson's Diversity index. Alpha diversity was calculated using the "Phyloseq" and the "microbiomeSeq" R packages. A one-way (accession or history) ANOVA and a Tukey's HSD post-hoc test were used to determine if alpha microbial diversity was significantly different between accessions and history groups. Additionally, beta diversity for accession and history was calculated using the Bray-Curtis dissimilarity method. The dissimilarity matrices were then analyzed with non-metric multidimensional scaling and permutational multivariate analysis of variance (PERMANOVA). All beta diversity analysis was conducted using the "Vegan" package in R. Furthermore, using the "Vegan" package in R, a redundancy analysis was performed to calculate the amount of variation present in species that can be explained by accession and history, respectively. To test for differences in the abundance of phyla and families, the "DESeq2" package in R was used. Lastly, to test for linear correlation between rotational values and microbial presence at the phylum and family level, the Pearson correlation coefficient was calculated for rotational values versus all normalized microbial groups using the "psych" package in R.

5.4 Results

5.4.1 PSF Values of Soil Measurements

The PSF values of soil chemistry measurements varied between modern cultivars, landraces, and wild peas, with modern cultivars and landraces generally having positive or neutral values and wild peas having negative or neutral values (Figure 1). Significant differences in PSF values for %N ($F_{2,50} = 4.492$, $P = 0.016$), %C ($F_{2,50} = 3.256$, $P = 0.046$), and manganese ($F_{2,50} = 3.301$, $P = 0.044$) were observed between modern cultivars, landraces and wild peas, with domesticated (modern cultivar, landraces) peas having higher PSF values than wild peas (Figure 1). Conversely, for potassium ($F_{2,50} = 2.295$, $P = 0.110$), the PSF value of wild peas was higher than modern cultivars; however, for both wild peas and modern cultivars, potassium PSF values were negative. Landrace potassium PSF values were neutral. Additionally, the aboveground biomass of wild and domesticated plants significantly affected soil rotational values for pH ($F_{1,50} = 6.988$, $P = 0.010$), potassium ($F_{1,50} =$, $P = 0.035$), and magnesium ($F_{1,50} = 7.801$, $P = 0.007$). A significant overall negative correlation between pH and total aboveground biomass ($r = -.263$, $t_{58} = -2.076$, $P = 0.042$) and a nearly significant negative correlation between magnesium and total aboveground biomass ($r = -.243$, $t_{58} = -1.907$, $P = 0.062$) were observed (Supplemental Figure S6).

Similar to domestication history, accession variation of soil PSF values were widespread, with accessions having positive, negative, or neutral values (Figure 2). Accessions varied significantly in PSF values for calcium ($F_{14,56} = 2.327$, $P = 0.013$), magnesium ($F_{14,56} = 2.829$, $P = 0.002$), manganese ($F_{14,56} = 2.269$, $P = 0.016$), sodium ($F_{14,60} = 2.517$, $P = 0.007$), effective CEC ($F_{14,56} = 2.360$, $P = 0.012$), and %C ($F_{14,53} = 5.259$, $P =$

< .001) (Figure 2). Additionally, the aboveground biomass of accessions significantly affected PSF values for potassium ($F_{1,50} = 5.754$, $P = 0.020$), even though potassium and total aboveground biomass were not significantly correlated (r = .193, $t_{58} = 1.498$, $P = 0.140$). Lastly, the homogeneity of variance between accessions and history were non-significant for all soil PSF values.

5.4.2 Recruited Soil Communities

Alpha (α)-diversity (richness, evenness, Simpson's, Fisher) and β-diversity (Bray-Curtis dissimilarity) measurements for both history and accession were non-significant (Supplemental Figure S1). However, the redundancy analysis revealed nearly significant clustering by history ($F_{3,62} = 1.063$, $P = 0.061$) with RD1 (55.7%) and RD2 (23.2%), which explains 78.9% of the variation found in the data (Supplemental Figure S2). Furthermore, differential abundance at the phylum and family levels for history and accession was non-significant for all phyla and families. Despite a lack of significant differential abundance between accession and history, significant differential abundances were found between history groups versus bulk soil for 5 phyla, with *Firmicutes* (t = -18.273, $P = <.001$) and *Patescibacteria* (t = -13.714, $P = <.001$) enriched in Pea rhizospheres, and *Chloroflexi* (t = -10.166, $P = <.001$), *Gemmatimonadetes* (t = -12.243, $P = <.001$), and *WPS-2* (t = -12.148, $P = <.001$) enriched in bulk soil (Supplemental Figure S3).

5.4.3 PSF and Rotational Values for Corn Measurements

The PSF values for wild and domesticated peas were widespread with positive, negative, or neutral values (Supplemental Figure S4). Despite the present variation between

wild and domesticated peas, rotational values for cob weight ($F_{1,50} = 3.036$, $P = 0.088$), vegetative weight ($F_{1,40} = 0.876$, $P = 0.355$), plant height ($F_{1,51} = 0.032$, $P = 0.859$), and chlorophyll content for newest ($F_{1,55} = 0.331$, $P = 0.567$) and oldest ($F_{1,55} = 0.122$, $P = 0.729$) leaf were non-significant between wild and domesticated peas. Similarly, accession corn PSF values varied with positive, negative, or neutral values. However, rotational values (cob weight) were significantly different between accessions ($F_{14,36} = 2.313$, $P = 0.021$), with accessions W6 26154 PSP (wild) and PI 577142 (domesticated) having the two highest rotational values (Figure 3). Vegetative weight ($F_{14,27} = 1.203$, $P = 0.328$), plant height ($F_{14,27} = 0.567$, $P = 0.874$), and chlorophyll content for newest ($F_{14,42} = 0.601$, $P = 0.849$) and oldest ($F_{14,42} = 1.161$, $P = 0.338$) leaf were non-significant between accessions. Additionally, the amount of corn present in each plot and the homogeneity of variance between history and accessions were non-significant for all corn measurements.

Rotational value was significantly correlated with a number of cover cropping measurements. Iron (r =.333, $t_{52} = 2.546$, $P = 0.014$) was the only PSF soil calculation that was positively correlated with rotational value (Supplemental Figure S6). Additionally, the total aboveground biomass (r =.357, $t_{52} = 2.785$, $P = 0.007$) of the plot was the only pea aboveground measurement significantly correlated with rotational value (Supplemental Figure S6). Furthermore, the presence of two phyla and nine microbial familial groups were significantly positively correlated with rotational value; *Epsilonbacteraeota* (r =.282, $t_{52} = 2.119$, $P = 0.038$), *BRC1* (r =.269, $t_{52} = 2.014$, $P = 0.049$), *Spirochaetaceae* (r =.388, $t_{52} = 3.093$, $P = 0.003$), *VC21 1 Bac22 Unclassified* (r =.349, $t_{52} = 2.682$, $P = 0.009$), *Mycoplasmataceae* (r =.349, $t_{52} = 2.682$, $P = 0.009$), *Arenicellaceae* (r =.349, $t_{52} = 2.682$, $P = 0.009$), *Limnochordales Unclassified* (r =.349,

t_{52} = 2.682, *P* = 0.009), *S47* (r =.349, t_{52} = 2.682, *P* = 0.009), *Demequinaceae* (r =.311, t_{52} = 2.360, *P* = 0.022), *Dadabacteriales Unclassified* (r =.309, t_{52} = 2.343, *P* = 0.023), and *Methylococcaceae* (r =.270, t_{52} = 2.023, *P* = 0.048).

Lastly, 16 families were negatively correlated with rotational values; *Leptotrichiaceae* (r =-.400, t_{52} = -3.150, *P* = 0.003), *CHAB-XI-27 Unclassified* (r =-.384, t_{52} = -2.999, *P* = 0.004), *WS6 (Dojkabacteria) Unclassified* (r =-.378, t_{52} = -2.943, *P* = 0.005), *Ellin5290 Unclassified* (r =-.355, t_{52} = -2.735, *P* = 0.009), *Neisseriaceae* (r =-.352, t_{52} = -2.711, *P* = 0.009), *Thermomonosporaceae* (r =-.351, t_{52} = -2.702, *P* = 0.009), *Bacillales Unclassified* (r =-.346, t_{52} = -2.657, *P* = 0.010), *SHA-37 Unclassified* (r =-.336, t_{52} = -2.575, *P* = 0.013), *Gemmatimonadales Unclassified* (r =-.329, t_{52} = -2.511, *P* = 0.015), *Balneolaceae* (r =-.327, t_{52} = -2.496, *P* = 0.016), *FAC88 Unclassified* (r =-.314, t_{52} = -2.389, *P* = 0.021), *Desulfobulbaceae* (r =-.312, t_{52} = -2.365, *P* = 0.022), *Armatimonadales Unclassified* (r =-.298, t_{52} = -2.504, *P* = 0.029), *Kouleothrixaceae* (r =-.278, t_{52} = -2.086, *P* = 0.042), *Geminicoccaceae* (r =-.277, t_{52} = -2.080, *P* = 0.043), and *Thermomicrobiaceae* (r =-.276, t_{52} = -2.073, *P* = 0.043)..

5.5 Discussion

The main aim of this study was to determine if variations in cover cropping traits and rotational value exist within field pea. Our data revealed that variation in cover cropping traits does exist within pea, with significant differences found between modern cultivars, landraces, and wild peas. Furthermore, when focusing on the accession level, significant variation was found in PSF soil measurements and rotational values. Therefore, our results indicate that the genotype of a cover crop could have a profound effect on soil

properties and the yield of a subsequently planted crop. However, this study's limitations must be considered, as this experiment took place at a single site over one cover cropping season. Therefore, gene-environment interactions and soil legacy effects, which have been seen to influence plant physiology, could have had an impact on our findings (Detheridge et al.; Wang et al.; Huang et al.). Whether the results obtained in this study were field-specific or universal has yet to be determined, and further long-term and multi-site experimentation are required. Despite these limitations, our findings are novel as they illustrate that crops could be improved as rotational partners, highlighting the use of wild relatives as a phenotypic reservoir for crop improvement.

Soil PSF measurements were significantly influenced by domestication with modern cultivars and landraces, as they increased the amount of macro- (C% and N%) and micronutrients (manganese) in the soil relative to the control plots (Figure 1). When focusing on the accession level, significant differences were also observed for macro- (C% and magnesium) and micronutrients (manganese, calcium, and sodium) between accessions (Figure 2). These results are not surprising since cover cropping field pea has been previously shown to increase the presence of macro- and micronutrients in soil, with legumes being proficient at increasing soil N and C (McDaniels et al., 2015). Additionally, Mwafulirwa et al. (2016) noted differences in C deposition for barley genotypes. However, this is the first time—to our knowledge—that differences in these benefits have been described for field pea. Overall, these results indicate that field pea could be potentially bred to improve its effect on soil properties in agroecosystems.

Recruited microbial communities did not differ in α-diversity between domesticated and wild peas at the history or accession levels. This was expected, as previous studies have shown a nonsignificant difference in α-diversity between CWRs and their domesticated counterparts (Pérez-Jaramillo et al., 2017; 2018). Additionally, β-diversity and differential abundance analysis revealed that pea rhizospheres of domesticated and wild accessions were non-significantly different from each other. These results were unexpected, as a previous meta-analysis revealed enrichment differences in differential abundances between wild and domesticated barley (*Hordeum vulgare)*, lettuce (*genus Lactuca*), common bean (*Phaseolus vulgaris*), and *Arabidopsis* (Pérez-Jaramillo et al., 2018). Pérez-Jaramillo et al. (2018) concluded that wild relatives' rhizospheres were enriched with *Bacteroidetes,* while their domesticated counterparts were enriched with *Actinobacteria* and *Proteobacteria*. The disparity in our study's results when compared to previous results could stem from differences in environments (Fierer et al., 2010) and land management practices (Qiao et al., 2017), which have been seen to have stronger effects on soil microbial communities than plant genotypes. Additionally, the lack of significance for β-diversity and differential abundances between pea accessions could have resulted from the limited number of accessions used in this study as it may not have fully captured the entire genetic or phenotypic diversity of microbial recruitment in field pea. Potentially, using a more comprehensive pea germplasm collection, such as the USDA Pea Single Plant Plus Collection, which contains 431 pea accessions that encompass the entirety of the world's genotypic and phenotypic pea germplasm collection, would be required to fully address this limitation (Holdsworth et al., 2017).

Despite finding nonsignificant differences for the microbial recruitment within pea, peas as a whole were enriched with distinct phyla when compared to bulk soil. Differential abundance analysis revealed that the pea rhizospheres had an increased presence of *Firmicutes* and *Patescibacteria* and a decreased presence of *Chloroflexi*, *Gemmatimonadetes*, and *WPS-2*. Finding an enrichment of *Firmicutes* in rhizospheres was not surprising, as *Firmicutes* have been associated with promoting plant growth (Mendes et al., 2013; Martins et al., 2015), disease suppression (Zhang et al., 2010; Mendes et al., 2013) and are predominantly found in plant rhizospheres in natural systems (Teixeira et al., 2010; Sarathambal et al., 2015; Wei et al., 2017) and agroecosystems (Zhang et al., 2010; Qiao et al., 2017; Gao et al., 2019). Additionally, *Patescibacteria* has been found in the rhizospheres of Andean maize (Correa-Galeote et al., 2016), Andean tubers (Chica et al. 2019), and sugarcane (Gao et al., 2019), with members of this phylum being a biological control for soil-borne pathogens in cotton (Zhang et al., 2011). Moreover, finding a lack of abundant *Chloroflexi* and *WPS-2* was not surprising, as these were not enriched in maize rhizospheres (Chica et al., 2019). Furthermore, *WPS-2* was only predominantly abundant in Cowpea (*Vigna unguiculata*) rhizospheres during senescence (Araujo et al., 2019). Chica et al. (2019) hypothesized that the lack of abundance of *Chloroflexi* and *WPS-2* in plant rhizospheres may be due to these phyla being oligotrophs (Koch, 2001; Fierer et al., 2007) and having the capability to utilize soil organic matter, including cellulose (Lauber et al., 2009; Bruce et al., 2010). Lastly, in *Populus*, *Chloroflexi,* and *Gemmatimonadetes* exhibited a positive and a negative correlation with the root exudate salicylic acid, respectively (Veach et al., 2019). The relatively low abundance of both of these phyla in

pea rhizospheres may indicate that the pea plants exuded an intermediate amount of salicylic acid.

Furthermore, the effect of accession and domestication history of a previously planted pea cover crop on a subsequently planted crop was limited, with nonsignificant differences found for plant height, chlorophyll content, and aboveground biomass. However, pea genotype did significantly influence rotational values (cob weight). Accessions W6 26154 PSP (wild), and PI 577142 (domesticated) had the two highest average rotational values (Figure 3). This may, in part, be due to these accessions having neutral and the second-highest PSF C% measurements, respectively (Figure 2). Additionally, accession W6 26157 PSP had the lowest rotational value and the lowest PSF Soil C% measurement. On average, legume cover crops have been shown to increase soil C by 24.5%, the highest soil C increase of all cover crops (Austin et al., in review). Moreover, long-term rotations, including pea and spring wheat rotations, increase total soil C and grain yields more effectively than other rotation combinations (Sainju et al., 2017). Most importantly, studies have shown that soil C is positively correlated with yields in agroecosystems (Lal et al., 2004; Sainju et al., 2017). However, in our study, PSF total soil C% was not positively correlated with rotational value. This may be due to the length of our study. Longer implementations of cover crops have been shown to have a more profound effect on soil organic carbon and soil organic matter, which contribute to total soil C% measurements (Olson et al., 2014; Poeplau & Don, 2015). Nonetheless, our results do suggest that the manipulation of soil total C% may have an integral role in determining the rotational value of accessions.

Rotational value was moderately positively correlated with several cover cropping measurements, one of which was the presence of iron. Iron is an essential micronutrient with strong effects on plant growth and yield due to it being a prerequisite for many cellular functions, such as photosynthesis, respiration, enzyme cofactors, redox reagent, and amino acid synthesis (reviewed in Kumar et al., 2017; Govindaraj et al., 2011). Therefore, a correlation between rotational value and iron was not surprising. Additionally, rotational value was moderately positively correlated with the presence of two phyla, candidate phylum *BRC1* and *Epsilonbacteraeota*. Accession W6 26154 PSP, the accession with the highest average rotational value, had also had the highest presence of *BRC1* (Supplemental Figure S5). *BRC1* may increase rotational value by suppressing diseases, as it has been significantly negatively correlated with wilt infection rates in tobacco (Yang et al., 2017; Niu et al., 2016) and has been consistently found on citrus leaves for trees that were asymptomatic for Huanglongbing disease (Zhang et al., 2013). In addition to *BRC1* and *Epsilonbacteraeota*, nine families were significantly positively correlated with rotational value. However, the majority of the normalized counts for these families were zero, and the correlations were primarily driven by a single sample, so these results should be taken cautiously. Nonetheless, *Spirochaetaceae* had the highest positive correlation with rotational value. Members of this family have been classified as pathogenic and can cause a myriad of diseases, such as syphilis, Lyme disease, leptospirosis, and swine dysentery; however, their effect on plants has not been thoroughly investigated (Karami et al., 2014). (Karami et al. 2014). *Mycoplasmataceae* was also positively correlated with rotational value. Some members of this family have been described as saprotrophs (Hurst, 2018); therefore, this family may increase rotational value through its enrichment of soil C

(Maaroufi et al., 2019). *Limnochordales Unclassified* was also positively correlated with rotational value. A previous study has shown that this family has been positively correlated with total phosphorus and potassium measurements in manure compost (Li et al., 2019). Soil testing of our field indicated high amounts of both phosphorus and potassium. Despite these relationships between cover cropping measurements and rotational values, we were unable to determine if these relationships were correlative or causational. Further experimentation that manipulates the absence and presence of these variables is required to address the true relationship between these variables and rotational value.

Cover cropping and crop rotation have been used in numerous agroecosystems throughout history to improve yields and soil quality. The results obtained from this study highlight the significant impacts of genotype on a cover crop performance. Implications from our research suggest that researchers studying cover cropping may now need to narrow to the genotype level rather than the family level (legumes, cereals, etc.) to facilitate agricultural production. Furthermore, CWRs should be utilized in cover cropping studies to reintroduce lost beneficial phenotypic and genotypic variation. In all, the results of this study suggest that cover crops can be improved as rotational partners to increase subsequently planted crop yields. Improving cover crop rotational values is imperative for sustainable agriculture and meeting the future nutritional needs of a growing human population.

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5.8 Figures

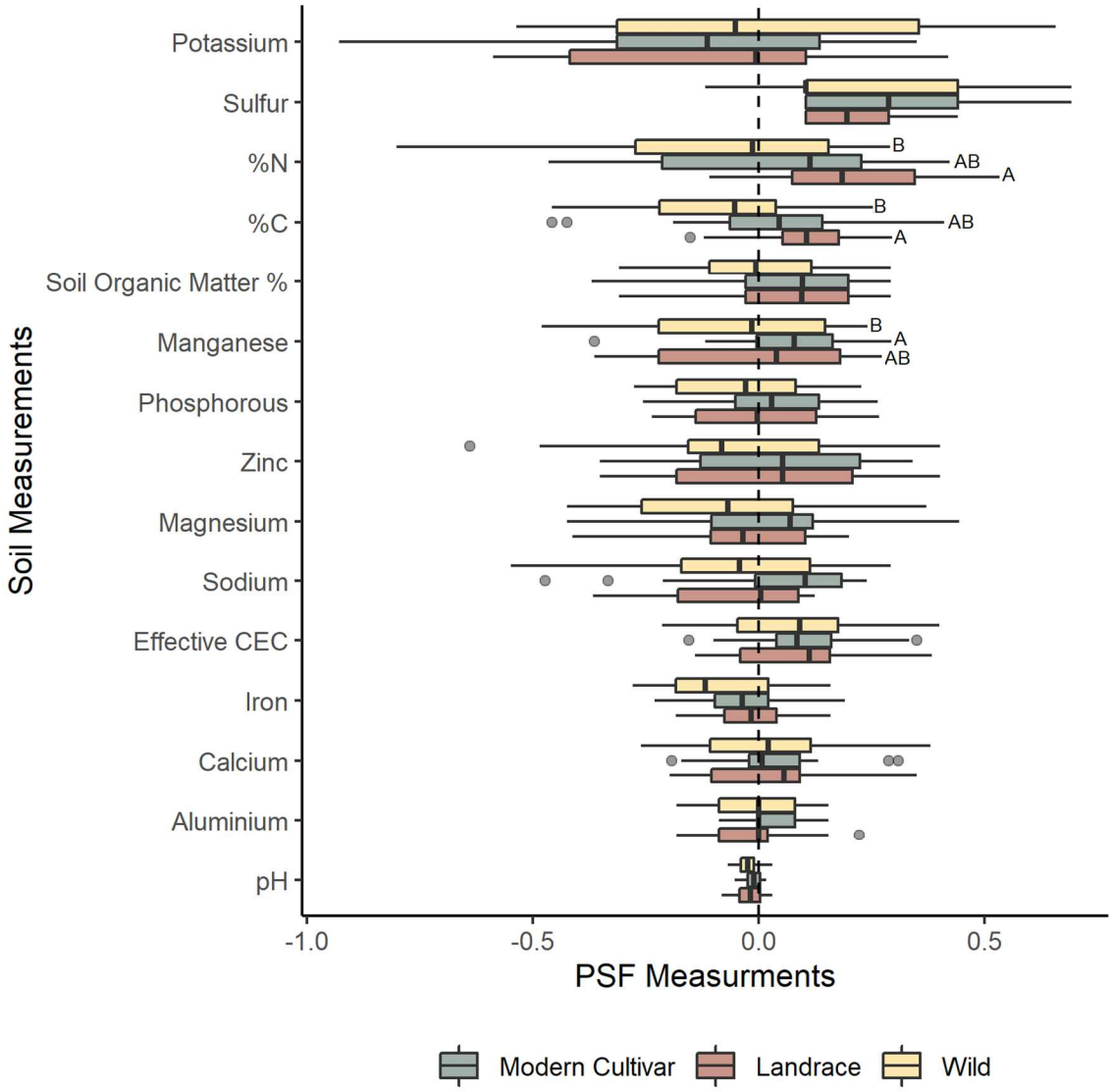


FIG. 5.1. PSF SOIL MEASUREMENTS BY DOMESTICATION HISTORY (MODERN CULTIVAR, LANDRACE, WILD). LETTERS INDICATE SIGNIFICANT DIFFERENCE WITHIN SOIL MEASUREMENT AT THE $P < 0.05$ LEVEL.

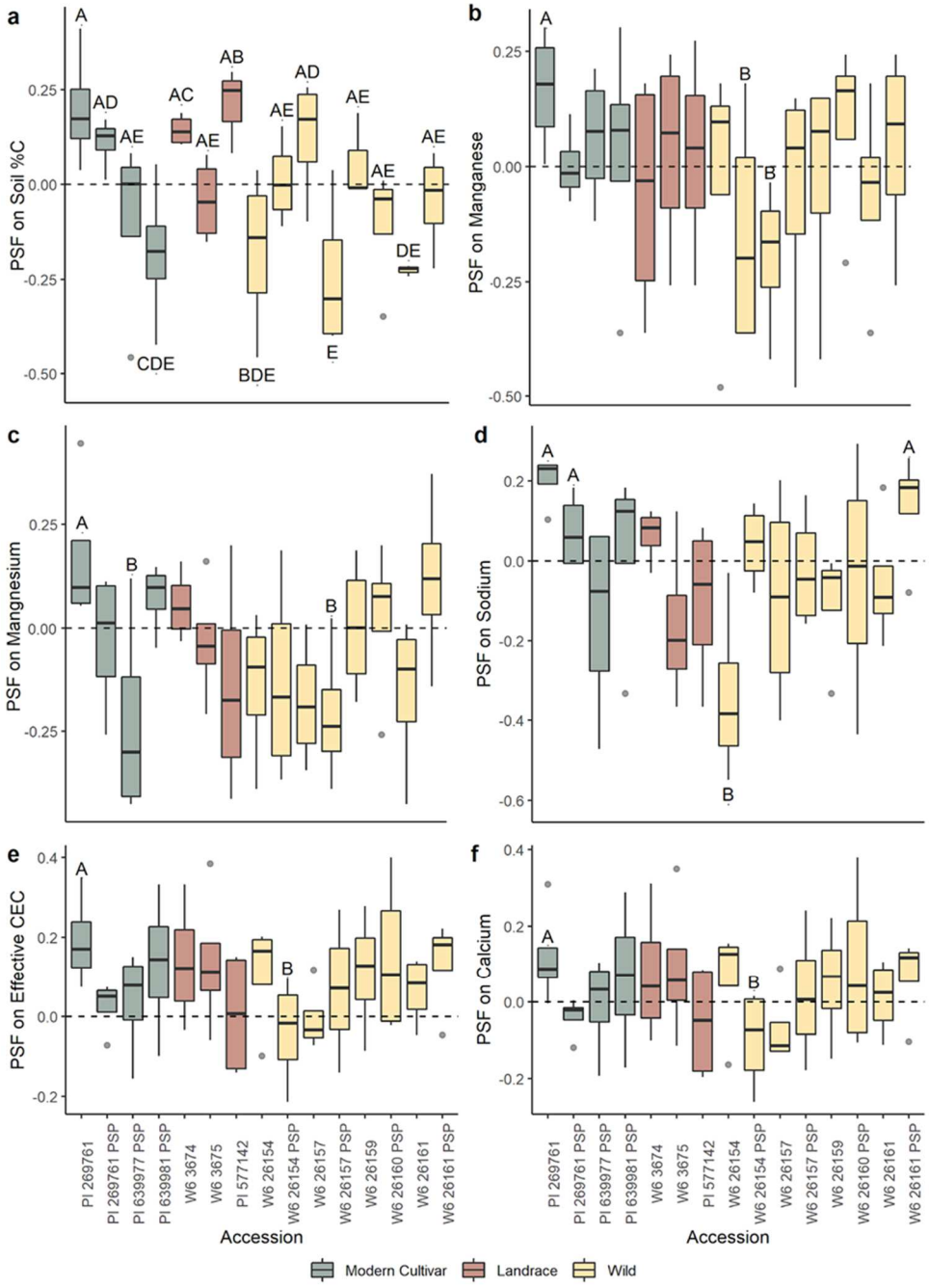


FIG. 5.2. PSF SOIL MEASUREMENTS BY ACCESSION (COLORED BY DOMESTICATION HISTORY: MODERN CULTIVAR, LANDRACE, WILD) FOR (A) %C, (B) MANGANESE, (C) MAGNESIUM, (D) SODIUM, (E) EFFECTIVE CATION EXCHANGE CAPACITY (CEC), (F) CALCIUM. LETTERS INDICATE SIGNIFICANT DIFFERENCE WITHIN SOIL MEASUREMENT AT THE $P < .05$ LEVEL.

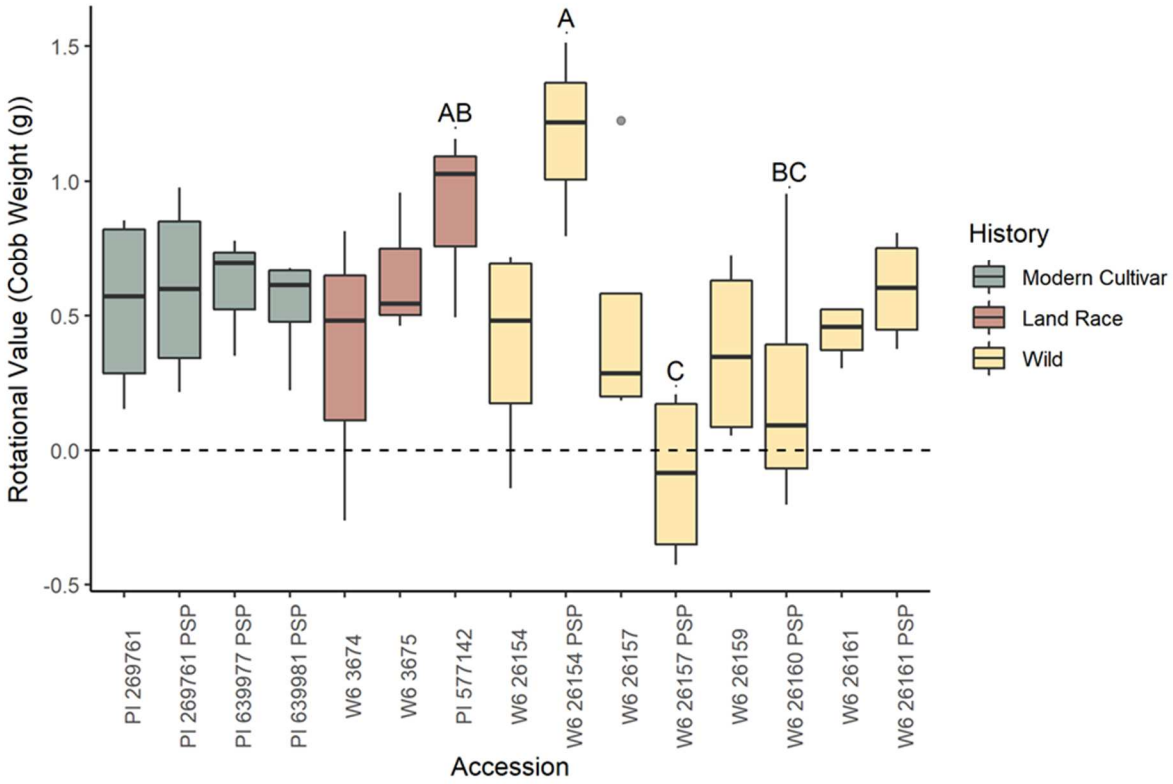
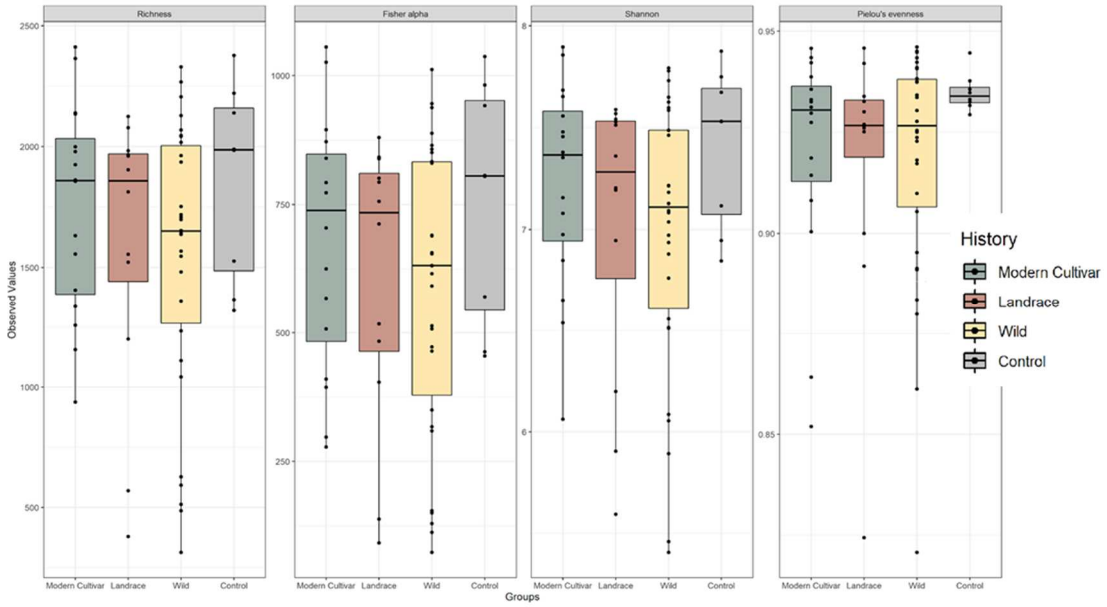
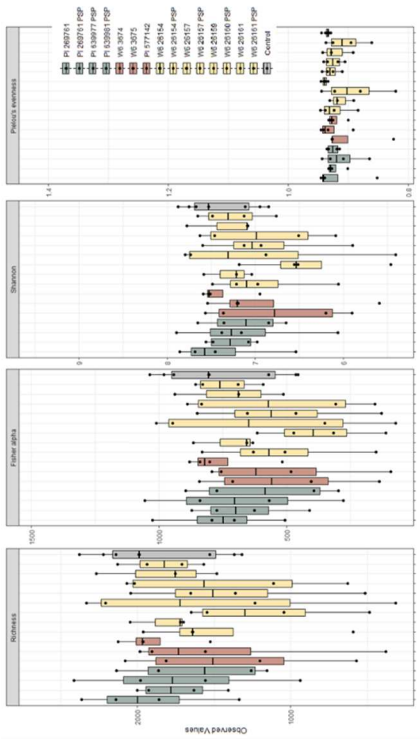


FIG. 5.3. ROTATIONAL VALUE MEASUREMENTS BY ACCESSION (COLORED BY DOMESTICATION HISTORY: MODERN CULTIVAR, LANDRACE, WILD). LETTERS INDICATE SIGNIFICANT DIFFERENCE WITHIN SOIL MEASUREMENT AT THE $P < 0.05$ LEVEL.

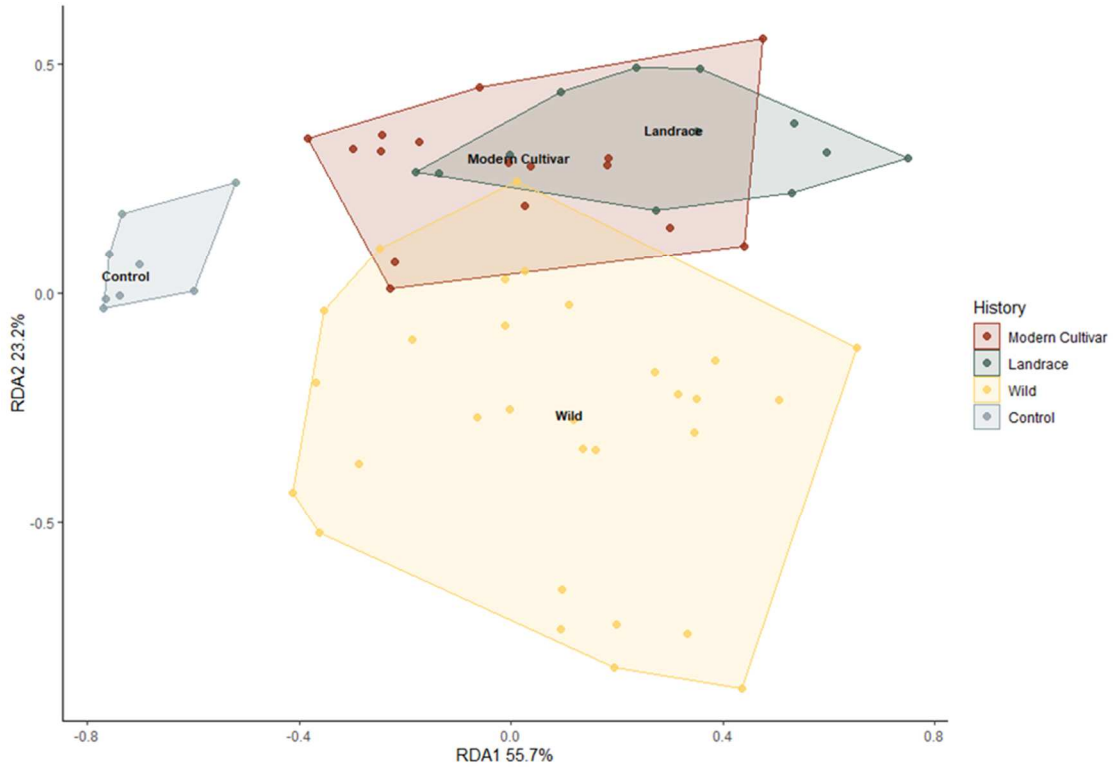
A.


Richness
Fisher alpha
Shannon
Pielou's evenness
Observed Values
Groups
Modern Cultivar
Landrace
Wild
Control
History

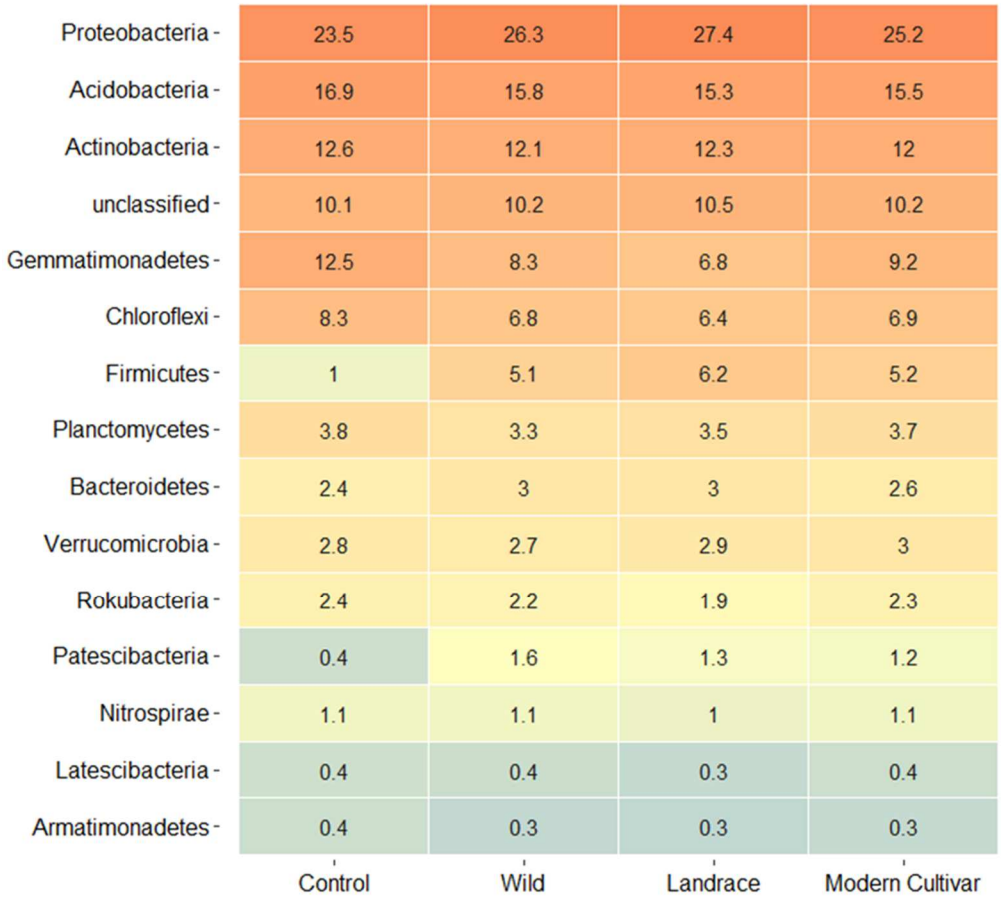
B.


Pielou's evenness
Shannon
Fisher alpha
Richness
Observed Values

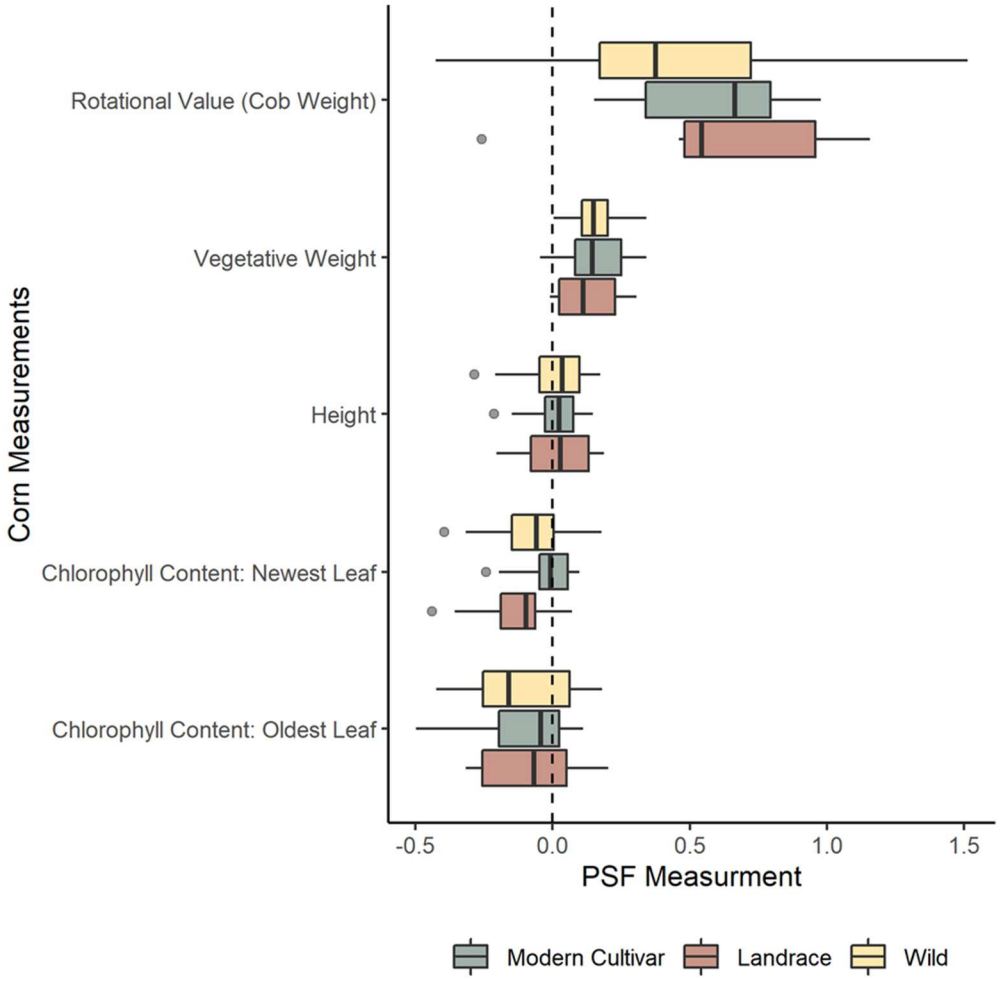
SUPPLEMENTAL FIGURE S1. ALPHA (A)-DIVERSITY (RICHNESS, EVENNESS, SIMPSON'S, FISHER) MEASURMENTS BY (A) DOMESTICATION HISTORY (MODERN CULTIVAR, LANDRACE, WILD) AND (B) ACCESSIONS.



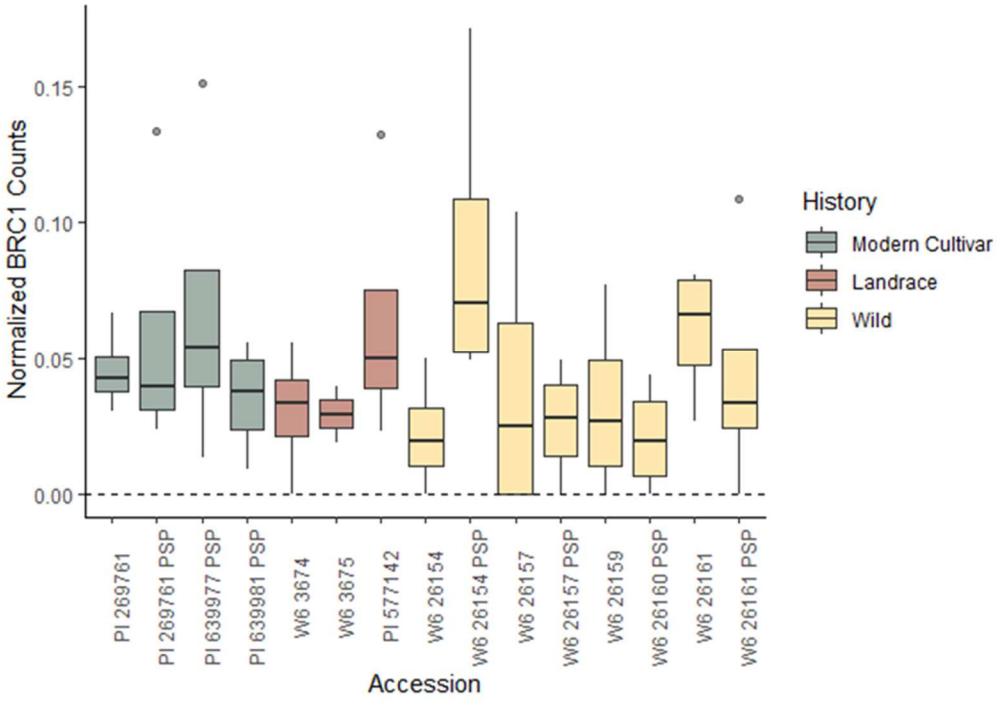
SUPPLEMENTAL FIGURE S2. REDUNDANCY ANALYSIS OF SPECIES COMPOSITION BY DOMESTICATION HISTORY (MODERN CULTIVAR, LANDRACE, WILD, CONTROL). RD1 (55.7%) AND RD2 (23.2%), EXPLAINS 78.9% OF THE VARIATION FOUND IN THE DATA.



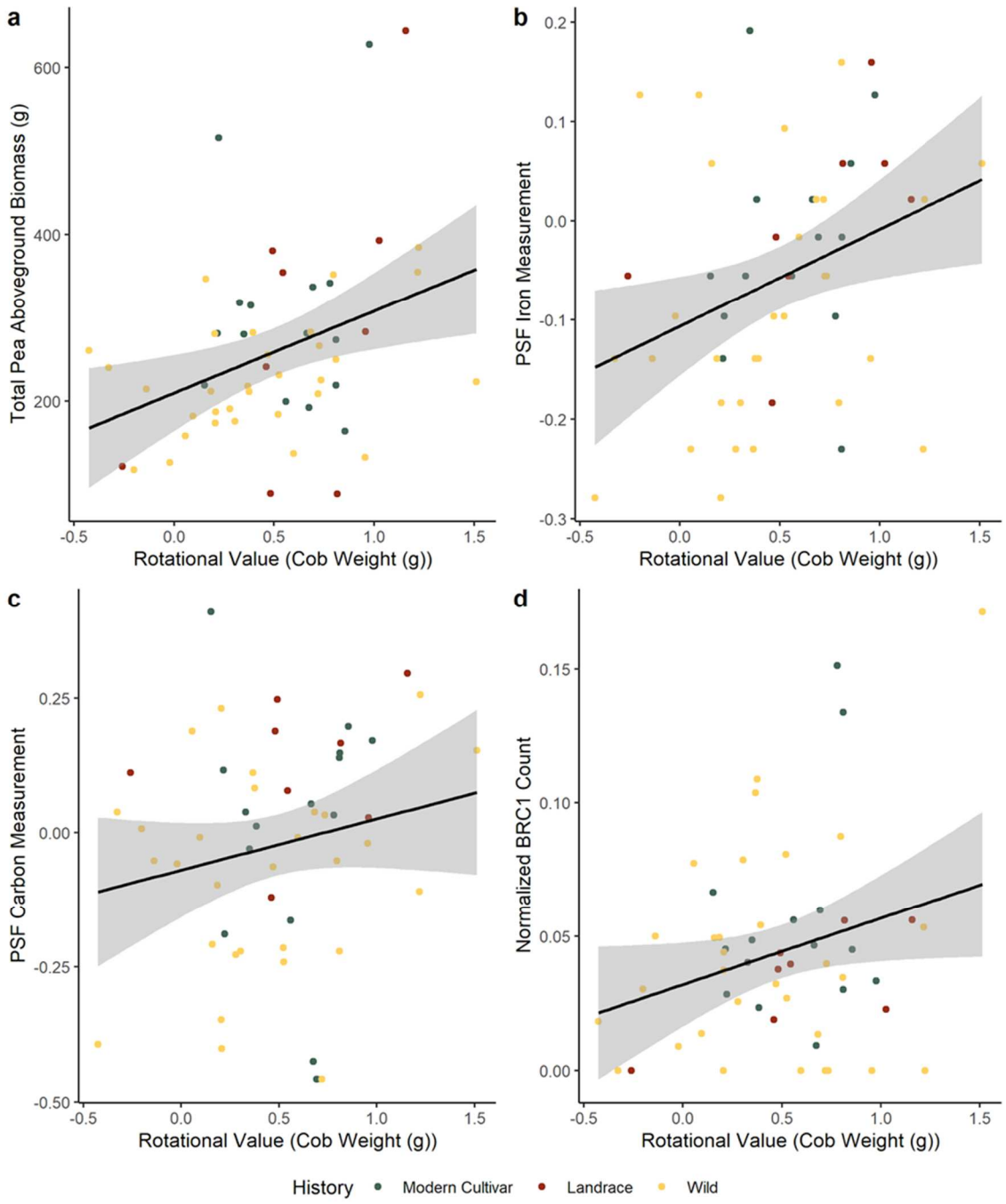
SUPPLEMENTAL FIGURE S3. RELATIVE ABUNDANCE OF THE 15 MOST ABUNDANT PHYLA BY DOMESTICATION HISTORY (MODERN CULTIVAR, LANDRACE, WILD, CONTROL).



SUPPLEMENTAL FIGURE S4. PSF CORN MEASUREMENTS BY DOMESTICATION HISTORY (MODERN CULTIVAR, LANDRACE, WILD). LETTERS INDICATE SIGNIFICANT DIFFERENCE WITHIN SOIL MEASUREMENT AT THE $P < .05$ LEVEL.



SUPPLEMENTAL FIGURE S5. NORMALIZED BRC1 COUNTS BY ACCESSION (COLORED BY DOMESTICATION HISTORY: MODERN CULTIVAR, LANDRACE, WILD). LETTERS INDICATE SIGNIFICANT DIFFERENCE WITHIN SOIL MEASUREMENT AT THE P <0 .05 LEVEL.



SUPPLEMENTAL FIGURE S6. CORRELATIONS OF ROTATIONAL VALUE BY A) TOTAL ABOVEGROUND PEA BIOMASS B) PSF IRON MEASURMENTS C) PSF CARBON MEASUREMENT D) NORMALIZED BRC1 COUNTS. POINTS COLORED BY DOMESTICATION HISTORY: MODERN CULTIVAR, LANDRACE, AND WILD ACCESSIONS,

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APPENDIX A: VERMONT WINTER PEA FIELD TRIALS

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Introduction

With 709 certified organic producers totaling 154,421 acres as of 2018, Vermont has one the highest per capita rate of organic farming in the United States (VT NOFA, 2020). Despite the popularity of fresh local produce in Vermont, local vegetable producers face significant challenges in maintaining soil fertility, reducing erosion, and nutrient loss (Myers et al., 2019). A sustainable strategy to combat these challenges is cover cropping, which is the method of planting a crop, not for its food production, but for its ability to manage soil erosion, soil quality, water, pathogens, and biodiversity (Reviewed in Hartwig and Ammon, 2002; Berendsen et al., 2012; Crews and Peoples, 2004; Connor, 2015; Reckling et al., 2016). The amount of cover cropped land in the United States has increased 49.7% from 2012 to 2017, and this dramatic increase can primarily be accounted to cover cropping ability to increase farm profitability and soil fertility while simultaneously lowering agriculture's impact on the environment (USDA, 2019).

There are many different species and types of cover crops for organic producers, but arguably, the most beneficial cover crops are legumes due to their ability to fix nitrogen, reduce pathogens, and promote soil carbon and microbial diversity (Reviewed in Baldwin and Creamer, 2006). However, due to Vermont's short growing season, there is insufficient time for vegetable producers to cover crop with legumes and produce their vegetables. One possible method to alleviate this problem is to cover crop during the winter season when vegetable production is not possible; yet, there are limited overwinter legume cover crops available that can tolerate Vermont's harsh winters. Hairy vetch (*Vicia villosa*) is currently the most popular conventional overwinter legume that can withstand these conditions but has severe drawbacks for organic farmers (CTIC et al., 2017). Hairy vetch

is hard to terminate using conservation tillage, such as chisel plows or disks, the most efficient method of terminating hairy vetch is a herbicide program using a mixture of glyphosate (Roundup) and dicamba (Banvel, Diablo, Oracle, and Vanquish) (PSU, 2010). Due to regulations and organic principles, the use of these herbicides is not feasible for organic producers, therefore, hairy vetch is difficult to terminate in organic settings.

A popular overwinter legume cover crop used by organic vegetable producers in other parts of the United States is winter pea (CTIC et al., 2016). In a recent CTIC et al. (2017) survey, field pea was listed as the second-highest planted legume cover crop in the US, with 42,355 total acres being planted by respondents. Winter pea is an ideal cover crop and green manure crop due to easy termination and its ability to break down quickly (SARE, 2015). Despite winter pea's potential benefits to our local organic farmers, currently, available winter pea varieties are unable to tolerate Vermont's winter conditions. However, newly developed cold-hardy winter peas by the USDA and Washington State University may provide the answer for a suitable organic Vermont overwinter legume cover crop (McGee et al., 2017). Therefore, in this study, we tested the viability of these newly developed winter pea varieties in Vermont by growing them over a single winter season at UVMs Horticultural Research Center. To test for viability we calculated emergence- and overwintering survivorship-percentage.

Methods

Plant Material and Experimental Design

Four plot replicates of ten cold-tolerant winter pea varieties, Specter, Windham, pss11300240w, ps11300289w, pss1430Nz003w, Lakota, Lynx, Koyote, Chelan, Blaze,

and a non-cold tolerant control, high mowing organic seed field pea (SKU 8070-A), were sown in a randomized design at the University of Vermont Horticulture Research Center in Burlington, Vermont on Sep, 18th 2018. Approximately 52 g of seeds were planted in 5.5^2 m plots at a depth of 1.27-2.54 cm. This was done to simulate the recommended cover cropping sowing rate of 85 pounds per acre. The control variety in this study was selected due to it being commonly used as a non-cold-tolerant cover cropping field pea by organic producers in Vermont. All cold-tolerant plant material was obtained from R. McGee, USDA-ARS, Pullman, Washington.

Emergence and Survivorship Measurements

After 14 days from sowing, the number of emerged plants for each plot was recorded. Then emergence percent was calculated by dividing the total number of the emerged plants in each plot by the approximate number of seeds planted in the plot. The approximate number of seeds planted in the plot was calculated by dividing 52 g by the weight (g) of 100 seeds for each variety and then multiplying it by 100. On May 1st, 2019, a week after daily low temperatures were consistently higher than 0□, we recorded the number of surviving plants (plants that showed new growth) in each plot. We then calculated survivorship percent by dividing the number of survived plants in each plot by the total number of emerged plants in each plot.

Statistical Analysis

A beta regression model and a likelihood ratio test were used to test for significant differences among accessions for emergence- and survivorship-percentage. A beta

regression model was used due to the data being bound between 0 and 1, which resulted in the data being non-normally distributed and/or heteroscedastic. Lastly, a Tukey's HSD post hoc was used to test for significant differences between accessions. All statistical analyses were performed in R (www.r-project.org).

Results

Emergence percentage was significantly significant for variety (X^2 (10) = 21.36, P = .0187); with Koyote (89.3%) having the highest average emergence percentage and ps11300289w (69.3%) having the lowest (Figure 1). However, in general, emergence percentages for the cold-tolerant varieties were not significantly different from each other, with the only exception of Koyote and ps11300289w (Figure 1). For survivorship percentage, varieties were significantly different from each other (X^2 (10) = 56.86, P = <.001). This was primarily driven by the difference in survivorship between the control and cold-tolerant varieties (Figure 2). As expected the non-cold tolerant control (0%) had the lowest survivorship percentage, despite having the fifth-highest germination percentage (84%). Cold tolerant varieties were non-significantly different from each other, however, survivorship percentages did vary somewhat (Figure 2). Lakota (43.9%), Windham (43%), and pss11300240w (41.9%) respectively had the highest survivorship percentage, while Specter (20.9%), Chelan (19.9%), and ps11300289w (9.7%) had respectively the lowest survivorship percentage (Figure 2).

Discussion

Overall, the data illustrates that five of the USDA developed cold-tolerant varieties, Koyote, Lakota, Lynx, pss11300240w, and Windham, are suitable for overwintering in

Vermont. Germination percentages for the cold-tolerant varieties were non-significantly different from the control, indicating that these varieties are adapted to Vermont's environmental conditions. However, this was not the case for ps11300289w, this variety had both the lowest emergence- (69.3%) and survivorship percentage (9.7%). Additionally, even though survivorship for five of the cold-tolerant varieties was significantly higher than the control, the survivorship percentages were still low, with the highest being 43.9% (Lakota). Furthermore, when taking into account the amount of seed planted, these survivorship percentages further decreased.

The results in the study were comparable to another winter-pea field study conducted in Eastern North Dakota (Johnson et al., 2007). The varieties Specter and Windham had respectively survivorship percentages of 15% and 40% in 2004/2005 and 91% and 92% in 2005/2006 (Johnson et al., 2007). Differences in survival percentage between these studies may be attributed to the previously planted crop. In Johnson et al. (2007) winter peas were sowed into hard red spring wheat (*Triticum aestivum* L.) standing stubble, while in this study winter peas were sown into a spring fallow field. The use of crop stubble may help facilitate overwinter survival by providing peas with a support structure to grow onto. Therefore, the use of a winter-hardy companion crop or the manipulation of previously planted crops could be a potential avenue to increase winter pea survival in Vermont.

In conclusion, our results provide necessary information regarding winter pea performance in Vermont and identified five potential varieties for Vermont overwinter production. However, additional replication of this study regarding locations and years is necessary before these varieties are recommended to producers. A second field trial is

currently underway with results expected by spring 2020. To increase winter pea survival two variables, time and depth of planting, have been changed from the original experimental design. Field peas were sown two weeks earlier than the previous year. We hypothesize with the additional time, peas may become more established to better withstand variable snow cover and freezing temperatures. Additionally, seeds were planted at a deeper depth, approximately 10.16 cm. This depth is approximately four times deeper than the previous trial. Collaborators at the USDA have shown that planting seeds at a deeper depth increases the survivorship of overwintering peas in the pacific northwest (Mcgee et al., 2017). This is most likely due to the soil acting as an insulator protecting the plants from the cold temperatures. These changes to planting time and depth may hopefully increase the survival of winter peas, which will bring us one step closer to a viable overwintering legume option for organic producers.

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Figures

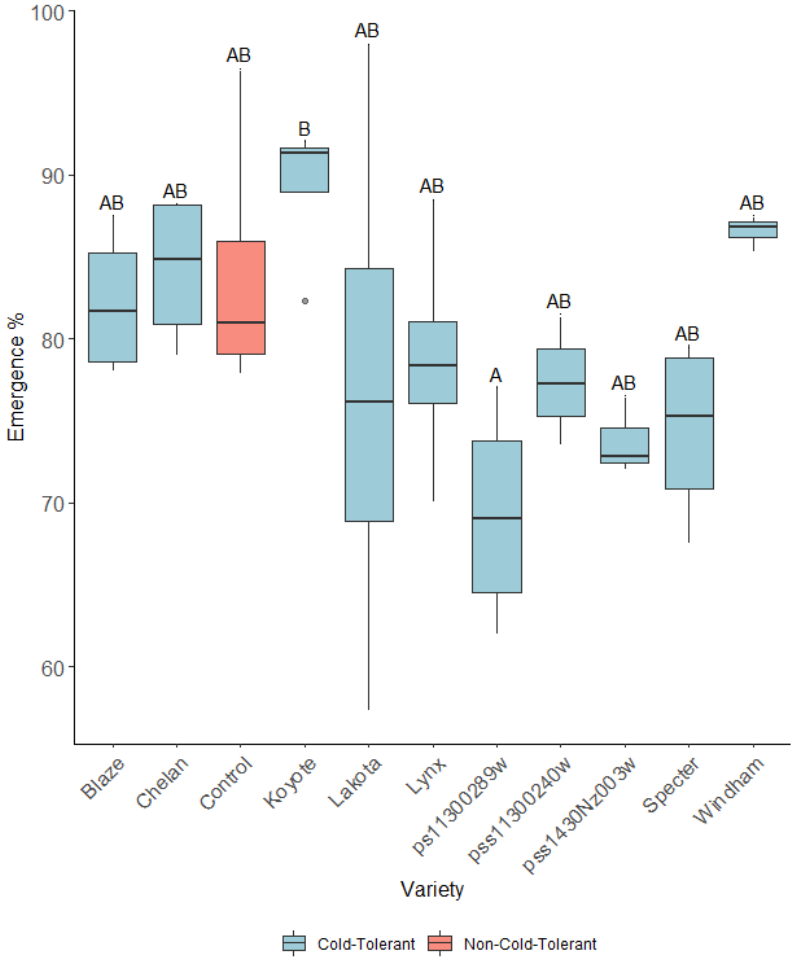

Figure A1. Boxplot depicting emergence percentages for 10 cold-tolerant varieties and a non-cold tolerant control variety. Letters indicate significant differences ($P < 0.05$) between varieties.

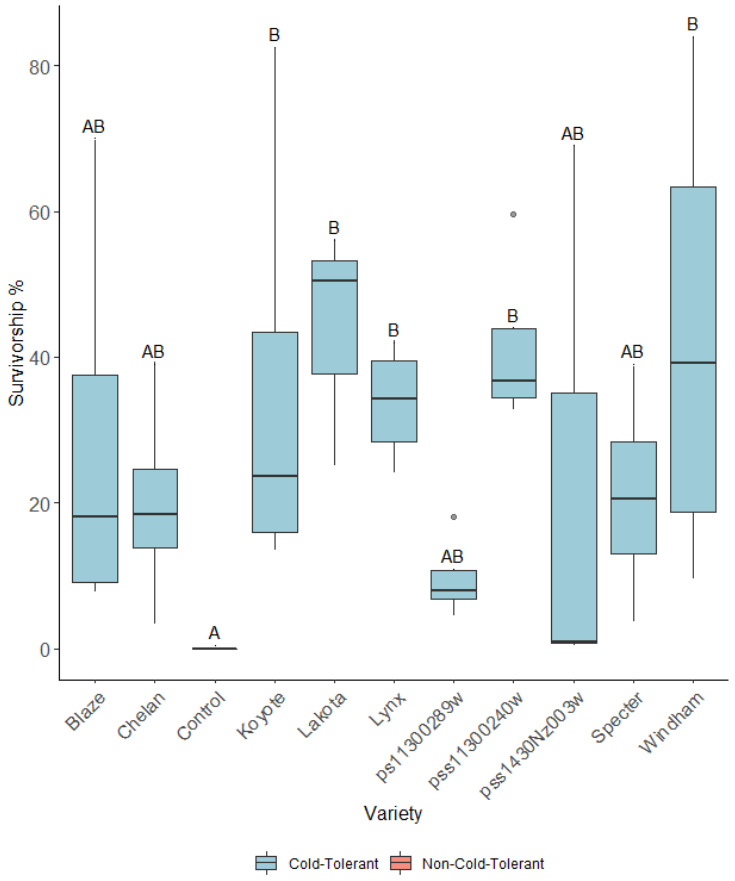

Figure A2. Boxplot depicting survivorship percentages for 10 cold-tolerant varieties and a non-cold tolerant control variety. Letters indicate significant differences ($P < 0.05$) between varieties.