

Article

Progress and Bottlenecks in the Early Domestication of the Perennial Oilseed *Silphium integrifolium*, a Sunflower Substitute

Alejandra Vilela ¹, Luciana González-Paleo ¹, Kathryn Turner ², Kelsey Peterson ²,
Damián Ravetta ¹, Timothy E. Crews ² and David Van Tassel ^{2,*}

¹ National Scientific and Technical Research Council (MEF-CONICET), Fontana 140, Trelew, Chubut, Argentina; vilela@agro.uba.ar (A.V.); lgonzalezpaleo@mef.org.ar (L.G.-P.); ravetta@agro.uba.ar (D.R.);

² The Land Institute, 2440 E Water Well Rd, Salina, KS 67401, USA, turner@landinstitute.org (K.T.); kelseyepeterson19@gmail.com (K.P.); crews@landinstitute.org (T.E.C.)

* Correspondence: vantassel@landinstitute.org; Tel.: +1-785-823-5376

Received: 22 January 2018; Accepted: 21 February 2018; Published: 28 February 2018

Abstract: Silflower (*Silphium integrifolium* Michx.) is in the early stages of domestication as a perennial version of oilseed sunflower, its close relative. Grain crops with deep perennial root systems will provide farmers with new alternatives for managing soil moisture and limiting or remediating soil erosion, fertilizer leaching, and loss of soil biota. Several cycles of selection for increased seed production potential following initial germplasm evaluation in 2002 have provided opportunities to document the botany and ecology of this relatively obscure species, to compare agronomic practices for improving its propagation and management, and to evaluate the differences between semi-domesticated and wild accessions that have accrued over this time through intentional and unintentional genetic processes. Key findings include: domestication has increased aboveground biomass at seedling and adult stages; seed yield has increased more, achieving modest improvement in harvest index. Harvest index decreases with nitrogen fertilization. Silflower acquires nitrogen and water from greater depth than typical crops. In agricultural silflower stands within its native range, we found that *Puccinia silphii* (rust) and *Eucosma giganteana* (moth) populations build up to unacceptable levels, but we also found genetic variation for traits contributing to resistance or tolerance. Breeding or management for reduced height and vegetative plasticity should be top priorities for future silflower research outside its native range.

Keywords: harvest index; yield components; specialist pests; N acquisition; new crops; breeding

1. Introduction

Investment in the development of perennial grains as future substitutes for similar annual grains has largely been justified by the expectation that the longer growing season, permanent root system and reduced frequency of tillage will slow or even reverse the loss and degradation of agricultural soils, use available resources more efficiently, and produce greater returns on investment of fuel and fertility [1–3]. For similar reasons, perennials are being sought as substitutes for annual bioenergy crops [4].

Perennials with coarse taproots may have additional advantages over other kinds of crops. First, they can use water stored deep in the soil thereby avoiding water stress during short-term droughts in rain-fed agriculture. In arid and semi-arid regions where irrigation is required for grain production, deeply rooted crops may require fewer irrigation events per year, reducing labor and surface evaporation.

Second, a crop extracting water from depth could acquire nitrogen along with the water. Nitrates leaching through the soil from nearby (or previous) crops normally become pollutants once they move

below the root zone of annual crops, resulting in both wasted nitrogen and contaminated groundwater. The perennial crop is said to provide a “root safety net” in this case [5,6].

Finally, there are situations where deforestation [7] or irrigation [8] are causing water tables to rise. A crop that can access water from the water table without requiring surface irrigation (after establishment) could help stabilize the hydrology of an agricultural area.

Silphium integrifolium Michx. (silflower, silphium, rosinweed) is in the early stages of domestication as a perennial oilseed grain and potentially a dual-purpose oilseed/biomass crop because it has naturally large seeds with quality and flavor similar to its relative the domesticated sunflower [9]. This genus is reputed to acquire water from below the rooting zone of many annuals and perhaps perennial grasses [10] and for that reason the target environments for early introduction of this new crop are likely to be those with unreliable soil moisture, nitrogen leaching, and irrigation problems.

Here we report examples of progress (or regression) in several general categories of research and development that are required for bringing any new plant into cultivation: (1) Improvements in understanding of basic crop biology, as required for predicting the potential geographical range and ecosystem services of the crop. Improved predictions about range and services, in turn, help improve breeding ideotypes and priorities; (2) Increases in the number and reliability of protocols for plant breeding, propagation, cultivation, harvesting, etc. make this species a more convenient experimental organism and make farmers more likely to participate in ongoing research; (3) Advances in cycles of artificial selection. Population means may have changed for traits under direct, deliberate selection but also for traits such as seedling vigor, which may have changed through unintentional selection or correlated responses to selection.

A series of experiments ranging from lab to field trials in Kansas (United States) and Patagonia (Argentina) were carried out in the last 15 years. Because of the particular objective of each experiment, the germplasm used included wild, unselected accessions as well as accessions that have undergone several cycles of selection for yield. These experiments also allowed us to evaluate other changes brought about by selection and to estimate progress and calculate heritability for some key characters. Base experiments to develop key agricultural practices such as germination and early seedling vigor, nutrient requirements and diseases are also reported. Taken together the disparate experiments discussed here capture much of the current state of the art of silflower cultivation and also reveal the gaps in our knowledge and deficiencies in the available plant materials where scientific attention should be focused as we move towards full domestication and adoption by farmers.

The Kansas location is close to the western limit of the native range of *S. integrifolium* and in a region with many native pastures and remnant prairies. Thus, agricultural plantations of silflower in this region cannot avoid *Silphium* pests and diseases. Although average rainfall in this region is sufficient for grain production, monthly and annual rainfall quantities are unpredictable and water deficits are common. Silflower is predicted to have greater yield stability than annual oilseeds in this environment.

Northern Patagonia’s irrigated valleys represent one of the other environments in which silflower may be able to offer unique agricultural alternatives to existing oilseed crops. While both Kansas and Patagonia environments have cold winters and hot summers, Patagonian valleys require irrigation for crop production, since precipitation is below 200 mm/year. Silflower’s deep-root system appears to tap into the water table located between 1.5 and 2 m deep, which could provide new options for reducing the environmental and economic cost of irrigation in this and similar regions.

2. Materials and Methods

2.1. Experimental Locations

2.1.1. Kansas

Research plots were located at The Land Institute’s experimental station near Salina (38°46’14” N; 97°35’30” W). Soil types on the research farm include Hord silt loam (fine–silty, mixed, superactive, mesic Cumulic Haplustolls) and Cozad silt loam (coarse–silty, mixed, superactive, mesic Typic

Haplustolls). The location is 370 m above sea level on the western fringe of the tallgrass prairie region. Mean annual precipitation in the area is 737 mm, with an average of approximately one-tenth of that precipitation coming in the form of snow. Rainfall is concentrated in spring and fall, and extended droughts are common in summer. The mean daily high temperature in July is 34 °C, the mean nightly low in January is −7 °C, and rapid, wide swings in temperature are common in all seasons. Predominant crops in the region are hard winter wheat (*Triticum aestivum* L.), grain sorghum, maize (*Zea mays* L.), and soybean [*Glycine max* (L.) Merr.].

2.1.2. Patagonia

Research plots are located at the Chubut River Valley (43°18'20" S; 65°19'15" W) near Trelew. Soil types of the farm include clay loam to clayish soils (self-mulching, inverting soils, rich in montmorillonite clay, typic Vertisols). These alluvial soils are slightly alkaline (pH 7.1), rich in organic matter 3.5% (0–10 cm), 0.286% total N, without salinity problems (0.53 mmhos/cm). The location is 10 m above sea level on the ecotone Monte-Patagonia (Cabrera, 1994). Mean annual precipitation in the area is 179 mm evenly distributed along the year. The mean annual temperature is 13.3 °C. The mean daily high temperature in January is 28.5 °C and the mean nightly low in July is 0 °C. Predominant crops in the region are horticulture and perennial pastures such as alfalfa (*Medicago sativa* L.), tall fescue (*Festuca arundinacea* Schreb), rye grass (*Lolium multiflorum* Lam.), and tall wheatgrass (*Thinopyrum ponticum* (Podp.) Barkworth & D. R. Dewey).

2.2. Plant Material

2.2.1. Botany

Silphium integrifolium (Asteraceae) is an herbaceous, long-lived diploid ($2n = 2x = 14$) perennial. It is indigenous to 21 states in the central United States and naturalized in Ontario, Canada [11]. To our knowledge, it is not naturalized or cultivated (outside of research and botanical gardens) anywhere else in the world. Achenes (dry, indehiscent fruits containing a single seed) are wind-dispersed, germinating rapidly after dormancy is broken by cold–moist stratification to form a rosette with profuse foliage growth. After one season in the field, and prior to flowering, internodes elongate (bolting). Erect stems (1–2 cm diameter) are sparsely clad with pairs of leaves, up to 15 cm long and quite variable in shape, ranging from lanceolate to ovate to elliptic and hairless to wooly. Inflorescences resembling small sunflowers appear in corymb-like clusters (5–7 cm diameter) in mid-summer. The aboveground tissues are somewhat frost tolerant but eventually die during the autumn. Shoot meristems overwinter as buds on short, shallow rhizomes. The rhizomes are woody and branching, forming a connected crown that slowly expands each year in diameter. Each crown has multiple coarse, vertical, persistent roots that can become woody. Abundant fibrous, horizontal roots can also be found in the spring, radiating out from the crown in the upper 10 cm of soil. These roots are not always found later in the year and may be ephemeral.

Tetraploid perennial sorghum (Poaceae): this new crop ($2n = 4x = 40$) was developed by backcrossing *Sorghum halepense* (L.) Pers. to *S. bicolor* (L.) Moench [12]. This grass perennates by spreading rhizomes but, in the breeding population described in this paper, not all genotypes produce rhizomes sufficiently cold tolerant to survive in Kansas. Aboveground tissues are very frost-intolerant and in these experiments, all aboveground biomass is shredded and left as a fine mulch each winter. Volunteer seedlings are common and difficult to distinguish from overwintering individuals except immediately after germination. Therefore, the sorghum plants in this study were likely a variety of ages.

Helianthus annuus L. (Asteraceae) is an annual oilseed grain crop. We obtained seeds of HA-89 and HA-292 from the USDA's Sunflower and Plant Biology Unit (Fargo, ND, USA).

2.2.2. Partially Domesticated Silflower Populations

In 1978, staff at The Land Institute planted 5-m rows of about 300 herbaceous, cold-tolerant perennial species [13,14]. At that time, the perennial sunflower relatives *Helianthus maximiliani* Schrad. and *Ratibida*

pinnata (Vent.) Barnhart were identified as promising candidates for domestication or experimental organisms due to questions about trade-offs between seed yield and lifespan [15]. *Silphium integrifolium* and *S. laciniatum* were two of the entries in the 1978 “herbarium” (Jackson, W. personal communication 1998) and a few individual silflower plants remain to this day in the approximate location of the original rows, although the field was allowed to “go wild”, probably in the 1980s. Most of the original 300 species disappeared or moved out of their original locations via rhizomatous spread or reseeding. The apparent longevity, non-spreading habit, drought and heat tolerance, and large seeds of both *Silphium* species inspired Land Institute staff to re-evaluate this genus [16]. Over 20 wild *S. integrifolium* (Table S1) and 50 *S. laciniatum* populations were located in central Kansas and seeds were collected in 2002 and grown for evaluation in 2003 (box A, Figure 1). *S. laciniatum* seedlings grew very slowly in 2003 and failed to flower in 2004. This species was deemed too difficult to establish and work with and was dropped from further experiments [16]. At this time, The Land Institute prioritized improvement of *H. maximiliani* and silflower domestication was carried on with minimal labor. For this reason, the 2003 germplasm nursery was used for seed increase and all seed was harvested and bulked together. Further cycles of selection are described in Figure 1. The primary goal in early years was to “feminize” silflower heads because, unlike sunflower, only ray florets are female-fertile. Disk florets produce only pollen. Because each ray floret has a single ligule (an elongated, showy petal), it was possible to rapidly locate individual plants (genets) with above-average numbers of ray florets per head. Some selection for achene mass and yield was also carried out between 2003 and 2015 (Figure 1).

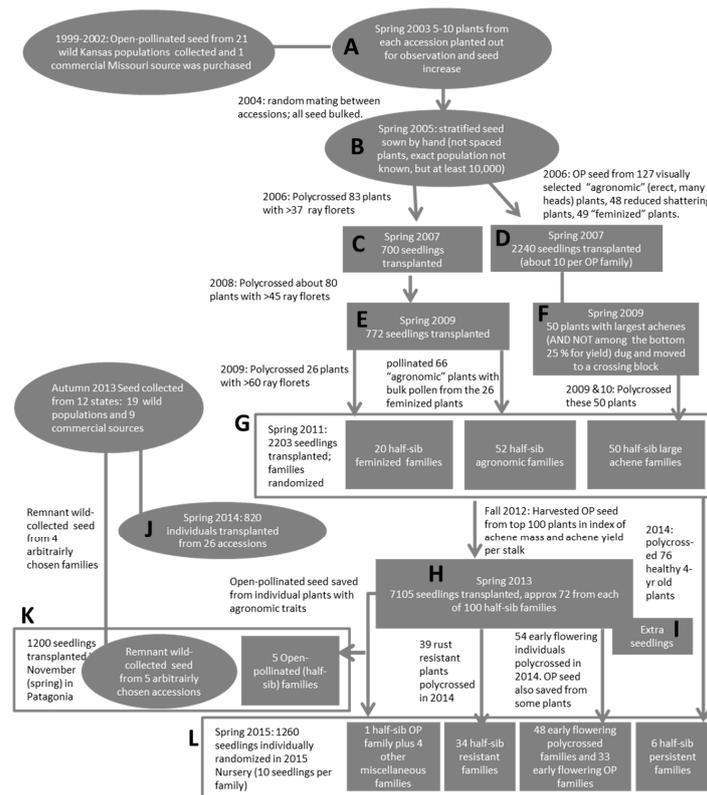


Figure 1. Relationship of the principal research populations in Kansas and Patagonia, 2003–2015. Populations with no history of artificial selection are shown in ovals; those that can be considered partially domesticated or “selected” are shown in rectangles. Populations connected by arrows are one sexual generation apart. Lines without arrow heads denote moving of seed or rhizomes between populations/experiments without a sexual generation. Population K was established in Patagonia, Argentina. All other populations were located in Kansas, United States. See Table S1 for details about the starting collections, Table S2 for population averages and heritabilities for traits in many of these populations, and Table S3 for explanations of traits mentioned here.

2.2.3. Wild Populations for Common Garden Experiments

The Land Institute acquired seed from many wild populations in several U.S. states in 2012–2013 by direct collection of seeds (bulking seeds from multiple adjacent plants in prairie remnants), or by purchasing seeds from regional native plant nurseries (Table S1). This seed was used to plant a large common garden in Kansas (population J, Figure 1). Four original accessions (two from Kansas; one each from Illinois and Minnesota) were arbitrarily chosen from accessions with sufficient remnant seed to generate seedlings used in a common garden experiment in Patagonia (experiment K, Figure 1). Leaves from these common gardens were sampled for resin content.

2.3. *Silphium* Biology and Ecology Methods

2.3.1. ^{15}N Enrichment with Soil Depth

On 23 June 2016, five healthy four-year-old silflower plants (population I, Figure 1) were randomly assigned to ^{15}N placement depths of 0.2, 1, 2, 3, or 0 (control) m in four 16 m² blocks. Plant spacing was 60 cm within rows and 91 cm between rows. Plants chosen for ^{15}N treatments were separated by a minimum of 2 m. Two holes of the assigned depth were augured for each plant, one 15 cm to the north and one 15 cm to the south of the crown. Augured soil was temporarily stored in a clean bucket. Each hole received ^{15}N enrichment as follows: A 1" PVC pipe was set into the augured hole and then lifted up approximate 10 cm. A 3/16" section of aquarium tubing was lowered down through the PVC pipe until it touched soil, at which time it was retracted by ~3 cm. Five milliliters of 98% ^{15}N -urea were pipetted into the tube followed by 25 mL DI H₂O. After ^{15}N and water were added, the tube was pulled up through the PVC pipe to avoid contact with soil, and then the PVC pipe was removed. Added solutions were allowed 20 min to soak in, at which time the holes were re-filled with augured soil in 50-cm increments that were punctuated with tapping by a blunt digging bar. Eighteen days after the enriched urea was placed in the soil, whole silflower plants including crowns but no roots were excavated. Entire plants were washed, dried, milled with a knife mill, sub-sampled, and pulverized with a ball mill. Care was taken to thoroughly clean mills between plants. Isotopic analyses of whole-plant ^{15}N were conducted by Kansas State University's Stable Isotope Mass Spectrometry Laboratory.

2.3.2. Leaf Water Potential

Tetraploid perennial sorghum plots of the same age and spacing were randomly interspersed with silflower plots, enabling comparisons to be made between the two species. Stem (silflower) and leaf (perennial sorghum) water potential measurements on two-year-old plants in the same plots described in Section 2.3.1 were made in 2014 using a PMS 1000 pressure chamber (PMS Instrument Company, Albany, OR, USA), following the approach of Boyer [17]. Sampling dates are given in Figure 2.

2.3.3. Fungal Pathogens

Taxonomy: To identify the species of rust infecting *Silphium*, we collected leaves with rust lesions in August of 2015 post-flowering from *S. integrifolium* and *S. laciniatum*. The samples were incubated in a moist petri dish at 37 °C for 48 h to germinate the teliospores. Teliospores were scraped from the leaf surface, transferred onto a glass slide, stained with iodine, and observed under a light microscope at 40x magnification. Two collections of dried rust-infected leaves were deposited in the Canadian National Mycological Herbarium ((DAOM) maintained by Agriculture and Agri-Food Canada in Ottawa, ON) with accession numbers DAOM 710575 (*S. integrifolium*) and DAOM 711883 (*S. laciniatum*). DNA sequences for the internal transcribed spacer region 2 region (ITS2) and partial 28S ribosomal RNA gene were generated using methods published in Demers et al. (2017) and deposited in GenBank as MG707785 (568 bp, *S. integrifolium*) and MG707786 (594 bp, *S. laciniatum*). Identification of the rust

as *Puccinia silphii* was based on morphological characteristics and DNA sequence comparison with collections of *Puccinia* spp. in the Canadian National Mycological Herbarium.

Leaf resin: Fully developed, healthy leaves of the same wild and selected accessions (see above) of plants growing in Kansas (population J, Figure 1) and in Patagonia (experiment K, Figure 1) were harvested at around full bloom. Leaves were oven dried at 60 °C and ground to 2-mm particles for Soxhlet extraction, with dichloromethane. Extracts were dried and the resin content was calculated on a per weight basis (% resin content).

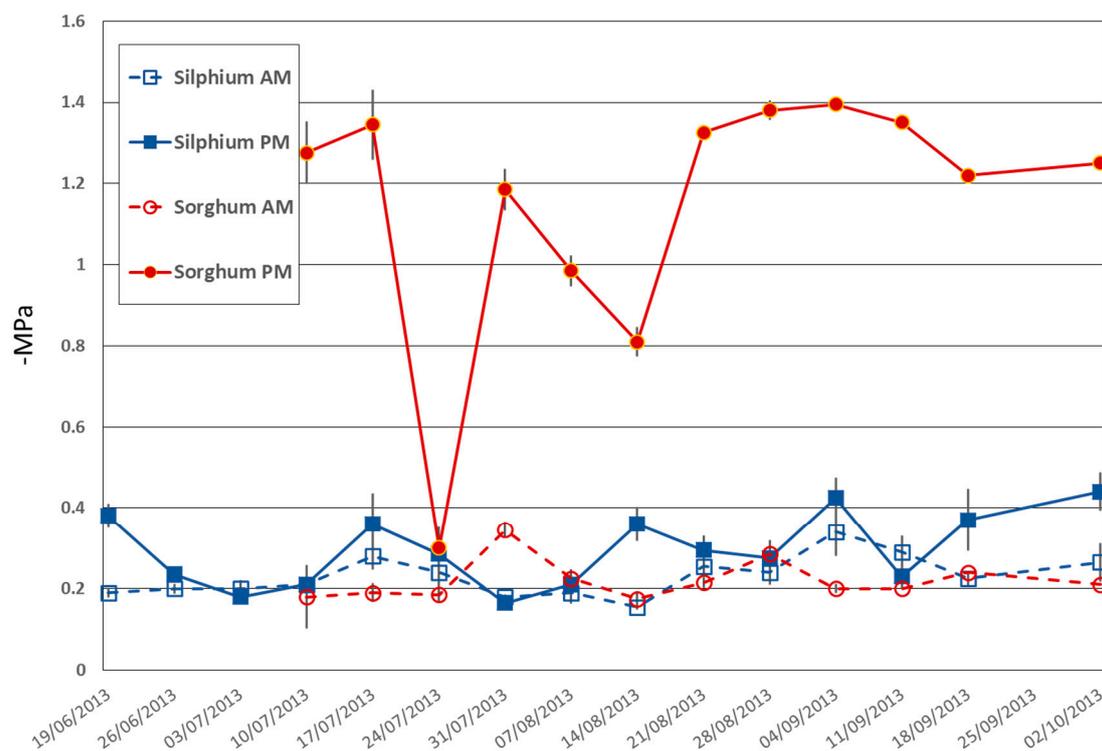


Figure 2. Summer pre-dawn and mid-day stem water potential of *Silphium integrifolium* and leaf water potential of perennial sorghum (*S. bicolor* *S. halepense*) grown in the same 0.5-hectare field. $n = 5$ for each crop species. *Silphium* sampling began on 19 July 2013 but sorghum sampling did not begin until 10 July 2013 because it emerges more slowly than *Silphium* in the spring. 24 July 2013 was wet and overcast.

2.3.4. Insect Damage

Larva-infested capitula: 220 immature capitula (inflorescences) showing clear signs of infestation (frass and or entry/exit holes visible) were labeled and monitored through the summer of 2017. These heads came from seven distinct plants (genets) growing in a single field (experiment L, Figure 1). Each infested head on two to three stalks (ramets) per genet was included in order to sample a representative range of head flowering times, positions, and sizes. We estimated capitulum size by measuring the diameter of the capitulum (including ray and disk florets but not the long yellow ligules). Capitula were collected when seeds had filled. Each capitulum was dried, and then hand threshed to determine the number and weight of filled carpels (achenes), number of empty or damaged carpels, and receptacle diameter.

Statistical analysis: We performed recursive partitioning to develop decision trees by implementing the conditional inference tree approach employed by the package ‘party’ in R v 3.3.2 [18,19]. A simple Bonferroni correction was used for the stop criterion with the nominal level of the conditional independence tests as $\alpha = 0.05$. The functions for classification and regression trees in the package ‘party’ guarantee unbiased variable selection when using the default parameters [18,20].

2.4. Agronomic Practice Development

2.4.1. Propagation

Asexual propagation: A factorial experiment was carried out to evaluate the effect of rooting media, concentration of a plant regulator, and the position of the cutting in the stem on rooting success and root formation. After bolting in early spring in Kansas (4 May 2016), 10-cm-long stems were cut from 50 plants and stored in plastic bags for transport to the greenhouse. Two types of cuttings were prepared from each stem: apical (upper three nodes) and sub-apical (nodes 4 to 6). A commercially available powder, indole-3 butyric acid (IBA) (Hormex, rooting powder, Brooker Chemical, Hollywood, CA, USA), was used in two concentrations, 0.1 and 0.8%. The cuttings were dipped in the IBA powder and placed in square pots (10 × 10 cm). Plant regulator was not used for control cuttings. Three rooting media were used: water, commercial potting mix (Promix Bx: peat moss; vermiculite; perlite; limestone, wetting agent and biofungicide), and soil:potting mix (1:1). A total of seven treatments (tap water; soil control; soil + IBA 0.1%; soil + IBA 0.8%; potting mix control; potting mix + IBA 0.1% and potting mix + IBA 0.8%; 10 reps per treatment; 5 cuttings per rep) were applied to each kind of cutting (apical or sub-apical).

Seed propagation: Silflower seeds are dormant at maturity and require cold moist stratification for germination, like other co-generic species [21]. In Kansas, the most common practice was to soak dry seed in 1 mM ethephon for 24 h, followed by rinsing and spreading seed on moist germination paper. After 3–5 days in a refrigerator (4–5 °C), papers were moved to 25 °C and seeds placed in 3.9 × 3.9 × 5.7 cm plug cells containing the commercial potting medium Pro-mix BX (PremierTech Horticulture, Quakertown, PA, USA) or Jiffy Preforma (Jiffy Products, Lorain, OH, USA) as soon as the radicle could be seen. Stratification of seed on germination paper for 4–6 weeks in the refrigerator is an alternative to the use of ethephon. In Patagonia, seeds were surface-sterilized with 1% sodium hypochlorite solution for 3–5 min, rinsed, and then stratified for 10–12 days in a refrigerator (4–5 °C) or until they germinated. Germinating seed was moved to germination trays with cells of 250 mL, filled with a 1:1 mix of sand and peat moss. Trays in both locations were maintained in a greenhouse and watered twice a day. Sixty days after emergence, seedlings were ready for transplanting in the field, typically in late May in Kansas and early December in Patagonia.

2.4.2. N Fertilization Experiment

A field experiment was carried out in Kansas, using two-year-old plants in a 0.6-ha field of silflower planted with seed harvested from breeding experiments (G and H in Figure 1). Seed was directly planted in September 2015 but did not germinate until the spring of 2016. We applied two N treatments: (1) N0—Control plants, without N addition; (2) N1—Fertilized plants, with the addition of 50 kg·N·Ha⁻¹. Nitrogen was applied once as urea to growing plants still in the vegetative (rosette) stage or early in the bolting stage. After three months of N application, 10 plants of each treatment were hand-harvested at physiological maturity (i.e., the head turning brown). We measured the number of stalks, total biomass, proportion of leaf senescence, total leaf area, number of lateral roots, seed yield, and yield components (seed weight, number of seeds per heads and number of heads per plant).

2.5. Breeding Methods and Breeding Progress Evaluation

2.5.1. Comparison of Wild and Selected Accessions of *Silphium integrifolium* Growing in Patagonia Argentina

Early vigor: Seedling relative growth rate was estimated using the paired plant method of Hunt et al. [22]. Briefly, 10 plants of each accession (five wild and five selected, see K in Figure 1) were randomly selected 60 days after seeding (9 December 2015). Pairs of seedlings of equivalent size and appearance were carefully removed from germination trays and the roots washed. Leaves and

roots of each plant were photographed with a digital camera to calculate total leaf area and total root length using the public domain ImageJ program. One seedling from each pair was transplanted to the field and the other destructively sampled by drying in an oven at 70 °C for 48 h and weighed with an analytical scale. The second seedling was destructively sampled 135 days later (24 April) and the difference in mass between the two time points was used to calculate RGR.

Common introductory garden in Patagonia: To compare growth and production changes brought about by selection for yield, a field experiment was carried out in the Chubut River Valley in Patagonia Argentina. Wild and selected accessions (five reps, experiment K in Figure 1) were compared after two years of cultivation. Seeds were sown in mid-September in germination trays filled with soil, peat moss, and sand in equal proportions and maintained in a greenhouse, where they received 80% of outside light levels and a temperature range of 25–15°C (average daytime/nighttime temperature). Seedlings were transplanted to the field 45 days after seeding (early December), in a completely randomized experiment with one factor, selection, and two levels: wild and selected (five accessions per level; five plots per accession, 24 plants per plot).

2.5.2. General Crossing Method (Kansas)

In July of multiple years, two to three stalks (of similar height) from each plant selected for crossing were tied together and any heads with any florets at or past anthesis were removed. Any heads with signs of insect damage were removed. Heads and leaves above or below the heads of interest were removed, leaving intact heads in a 30-cm zone at the top of the stalks. Yellow ligules were cut back to the base of the florets. Stalks were covered with a single cotton sleeve of 30 cm × 60 cm. The sleeve was securely tied below the heads. The top opening was folded over and secured with a clothes peg or similar clip. Every one to three days, the sleeve was opened at the top and folded down to expose the heads. Pollen was harvested from disk florets with elongated styles. Each morning, a new whorl of disk floret styles emerges through the ring of anthers and pick up pollen. Pollen may be harvested after mid-morning on warm days or after noon on cool, cloudy days. Water is thought to damage pollen, so pollen was never harvested during or immediately after rain.

Pollen was harvested by inverting each head over a small jar and gently brushing the styles with a clean hard object such as a wire or the blunt side of scissor blades. To keep pollen dry, detached petals and styles should be removed from the pollen as quickly as possible. We used a jar with small holes (1–2 mm in diameter) nested within an unperforated jar to immediately sieve the pollen. For polycrossing, pollen from each plant in the group was harvested into the same jar. For pairwise crossing, separate jars are used for each plant.

Following pollen harvest, emerged styles and anthers were generally excised to reduce the amount of litter in the next pollen harvest and to make it easier to see the fertile stigmas. For the same reasons, ligules were trimmed until they were barely longer than the fertile stigmas. Fertile stigmas are found only on the ray florets and, unlike the infertile styles of the ray florets, are bifurcated. Pollen was transferred to the stigmas using a pipe cleaner. Care was taken to apply pollen to the earlier whorls of ray florets whose stigmas are often behind the ligules of later whorls. After pollination, the cotton sleeves were rolled back up over the heads and resecured.

When polycrossing, the same pipe cleaner can be used to pollinate all plants in a group. For other types of crosses, we use 3-cm pieces of pipe cleaner and discard them after use.

Pollen was sieved with a tea-strainer, if necessary, and stored overnight in a cool, dry place. We have not studied pollen viability, but we generally leave pollen in an air-conditioned office. In dry climates a refrigerator can be used, but care must be taken to avoid condensation forming when moving pollen jars from the refrigerator to the outdoors.

2.5.3. Harvesting, Seed Cleaning (Kansas)

Open pollinate seed was collected by tying together one to three stalks and covering the heads with a nylon mesh bag (30 cm × 50 cm) after all larger heads have completed anthesis. Mesh bags

prevent bird predation and losses due to seed shattering. Bags were collected by cutting the stalk below their base after the heads inside were brown. Bags were allowed to finish drying indoors. Bag contents were removed and threshed by hand or using a belt thresher (model BT14, Almaco, Nevada, IA, USA). Pieces of stalk were removed by hand or using sieves, and smaller material was removed using a vacuum column separator (model STS-WM2, Seedtech, Wilton, CA, USA).

3. Results

3.1. Progress in Characterizing the Biology and Ecology of Silflower

While goals for improving standard domestication traits relating to seed production, harvesting, and processing are clear, defining a whole plant breeding/domestication ideotype for silflower must take into account the target regions and production systems (e.g., irrigated, unirrigated, organic, etc.). Defining target environments, in turn, requires knowledge of the distinctive stress adaptations and potential ecosystem services of this relatively unstudied genus [9]. In particular, we wanted to begin to understand how silflower acquires water compared with existing crops.

Developing management and/or genetic solutions to serious insect and pest problems, like breeding for non-shattering seeds, is standard procedure in crop breeding and domestication. However, for new crops such as silflower, little was known about which pests would be particularly severe within production plantations. Here we report some of our early insights that will shape future breeding goals.

3.1.1. Evidence of Distinctive Water Use Strategy

Leaf Water Potential

We compared the pre-dawn and mid-day stem water potential of several silflower plants every 15 d throughout the growing season. Comparisons with sorghum were made between 10 July and 2 October after perennial sorghum had emerged and reached sufficient size. While seasonal differences were not seen, Figure 2 shows that silflower stem water potentials are diurnally much more stable than perennial sorghum leaf water potential. Pre-dawn water potentials are similar for both crops.

This pattern suggests that sorghum, a drought-tolerant C4 grass, frequently experiences water deficits by mid-day, while adjacent silflower plants only experience mild imbalance between water loss and water acquisition. Both species were growing rapidly and achieved similar height and stand density. We infer from this that silflower is able to acquire soil moisture more rapidly than sorghum during the day. Larger diameter root xylem vessels in deep roots would explain this difference, although we do not have direct evidence for this.

¹⁵N Uptake at Depth

If silflower is able to rapidly translocate and transpire water from deep in the soil (as suggested by the results in “Diseases”), bulk flow of water may also provide an efficient route for the uptake of dissolved nitrogen found below the uptake zone of other crops. If this is true, silflower could provide a “root safety net” [5] to intercept nutrients leaching past intercrops, or help to remediate soil with excessive nitrogen. To provide preliminary evidence for this ecosystem service, we followed the approach of Kristensen and Thorup-Kristensen [23], supplying plants with dissolved ¹⁵N-enriched urea nitrogen fertilizer by carefully burying it at different depths and comparing the isotope enrichment of aboveground plant material with the isotope signature of control plants receiving no isotope (Control, in Figure 3).

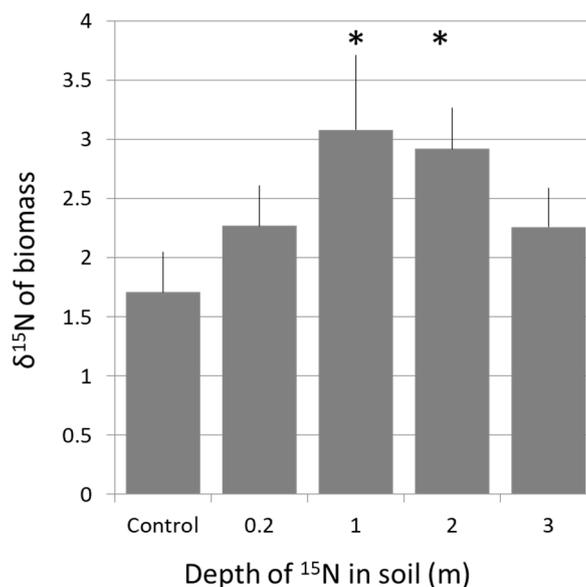


Figure 3. Enrichment of above ground *S. integrifolium* biomass with enriched ^{15}N -urea buried at four depths. Bars with * are significantly different from the control (one-tailed t-test, $p < 0.05$). Error bars show the standard error.

Our expectation was that plants would take up more ^{15}N from superficial applications than deeper ones because most plants have more superficial roots than deep roots. Unexpectedly, uptake was higher at 1 and 2 m than at 0.2 m. We cannot fully explain this result. Root density was found to be linearly related to ^{15}N uptake for several other species, and both diminished with depth [23]. Perhaps superficial roots are shed during the summer, or perhaps they radiate out from the base of a plant and primarily take up nutrients at some distance, which would mean that neighbor plants might have taken up the ^{15}N in these experiments. Neighboring plants may also have intercepted some of the nitrogen applied at the greatest depths, since tap roots are not vertical and instead angle out from the crown to form a (hollow) cone-shaped root zone [16]. At a minimum, these results demonstrate that silflower is capable of acquiring nitrogen from at least 2 m deep. Because nitrogen is often taken up through bulk flow of water, these results also support the emerging model that silflower acquires a large fraction of its moisture from 1 to 2 m depth and possibly deeper.

3.1.2. Pathogens and Herbivores

Although many *Silphium* species are long-lived and generally stress-tolerant, they are associated with a number of generalist and specialist parasites/predators. Relatively few insect pests and diseases were observed in research plots in Kansas between 2003 and 2013. This is still the case in Patagonia. However, in Kansas, rust spread rapidly in a single plot in April 2014. Plants were spaced more densely than normal in this plot (box I in Figure 1), and there was a rainy period preceding the outbreak. By June, rust pustules could be found on the plants bordering other silflower plots, presumably the result of spores spreading by wind. After this date, rust has become ubiquitous at The Land Institute's research farm, although it has never infected plants growing in the greenhouse during the winter. Insect herbivores have also become numerous and have begun to cause serious yield loss [24].

Diseases

Silphium species are infected by many bacterial and fungal diseases. Leaf spots are caused by the bacteria, *Pseudomonas syringae* pv. *tagetis*, and the fungi, *Ascochyta compositarum*, *Cercospora silphii*, *Colletotrichum silphii*, and *Septoria alba*; rusts are caused by *Puccinia silphii*, *Coleosporium terebinthinaceae*, and *Uromyces silphii*; powdery mildew is caused by *Erysiphe cichoracearum*; downy mildew is caused

by *Plasmopara halstedii*; root rots are caused by *Phymatotrichum omnivorum* and *Rhizoctonia solani*, and white smut is caused by *Entyloma compositarum* [25]; *Alternaria alternate* is known to infect *Silphium* stems, producing oblong brownish-purple lesions on the stems [26].

Of these diseases, rust has been the most prevalent disease since 2014 in central Kansas, although powdery mildew, downy mildew, and stem spotting have also been observed. Rust is a significant disease for many crops including cereal grains and annual sunflower, *Helianthus annuus*. In annual sunflower, *Puccinia helianthi* can cause premature leaf senescence [27] and reduced yield and oil seed quality [28]. *Puccinia* species are variable in the size of their host range and also in the number of spore stages in their life cycles. The type of spores produced, influence the dispersal mechanism, genetic diversity, and inoculum source. Identifying the causative species is important for designing inoculation and management techniques and planting the host in polycultures containing multiple plant species.

The rust species infecting *S. integrifolium* (and *S. laciniatum*) in Salina, KS was preliminarily identified as *P. silphii* based on its teliospore morphology and absence of other life cycle stages. The teliospores in our samples were orange–brown in color, with a narrowly ellipsoid shape with tapered ends with a slight constriction at the septum, and measured 33–51 × 15–20 μm with a pedicel length ~50 μm (Figure 4), which was consistent with the descriptions of *P. silphii* by Arthur [29] and Parmelee [30]. The sequences of the *P. silphii* accessions collected in Salina, KS have been deposited in GenBank as MG707785 (568 bp, DAOM 710575) from *S. integrifolium* and MG707786 (594 bp DAOM 711883) from *S. laciniatum* and can be publicly accessed. The sequences for each collection were identical and matched unpublished reference data for additional herbarium collections of *P. silphii* [31]. These sequences will be useful in our future research to design primers for identification and quantification of the species. Other rust species including *Coleosporium terebinthinaceae* and *Uromyces silphii* are known to infect *Silphium* spp., but were not observed in our samples. Unlike the rust that infects annual sunflowers, *P. helianthi*, *P. silphii* is microcyclic, lacking the uredinia fruiting bodies that produce a large number of spores easily transported by wind. If the destruction of teliospores can be achieved through management, it may be possible to significantly reduce the inoculum load.

We found that the two *H. annuus* lines, HA-89 and HA-292, planted as checks in fields containing silflower, were not infected by *P. silphii* under high disease pressure. Since the host range of *P. silphii*, *C. terebinthinaceae*, and *U. silphii* do not include *H. annuus*, the potential for *Silphium* fields to act as a reservoir of inoculum for annual crops is unlikely for rusts.

Anecdotally, we have observed an increase in the level of rust in our breeding plots, in contrast to wild *Silphium* populations in adjacent field margins, which have much lower levels of rust. The levels of rust may be due to a higher population density of *Silphium*, higher humidity, or higher nutrient availability to the host or pathogen in the agricultural plots. This represents a challenge in growing large plantings of *Silphium* species.



Figure 4. *Puccinia silphii* teliospores stained with iodine and magnified by 40×.

Insects

Although silflower nurseries appeared to be relatively pest-free between 2003 and 2013, annual observations since then by USDA entomologist Jarrad Prasifka suggest a growing population of invertebrate pests in silflower breeding plots in Kansas [32]. In 2017, the giant eucosma moth (*Eucosma giganteana*), hereafter referred to as “eucosma”, infested 95% of heads by late July, as shown by weekly examination of randomly selected heads. The giant eucosma moth’s life cycle revolves around a silflower host, utilizing silflower crown, stalks, and inflorescences as food and shelter.

The larval or caterpillar stage of eucosma largely occurs in the silflower capitula, where the caterpillars feed on male florets, receptacle, and developing seeds, usually destroying much of the inflorescence. However, we noticed that some seeds in infested capitula escape damage. These heads are heavily damaged, but in terms of seed production, we classify them as infested but tolerant. The large head-to-head variation in tolerance could be due to variation in insect behavior, or unpredictable interactions between insects and weather. Capitulum traits could also influence the pattern of seed escape. Because capitula vary greatly in size, position, and phenology within a single stalk, we hypothesized that they might also vary in qualities that influence larval growth or behavior and therefore the proportion of seeds damaged. Capitulum quality could also vary due to differences between plants.

Discerning patterns of seed escape would provide clues for developing strategies for breeding or management to improve crop tolerance to eucosma infestation. Therefore, we documented the number of intact seeds in infested heads from several different genets, and with varying capitulum and receptacle diameters, flowering times, and locations (main stalk vs. low side branch, basal vs. distal heads within the corymb-like clusters of heads).

We found wide variation in the number of seeds per capitulum that escaped predation, (ranging from 0 to 38, with a mean of 8.72) and capitulum diameter (ranging from 5.59 to 29.0 mm, with a mean of 15.5 mm). Among the five predictive variables, disc diameter is the covariate showing the most significant association with seed escape ($p < 0.001$). Two groups are distinguished, those with disc diameter greater than 17 mm ($n = 76$) have significantly more seeds than those with less than or equal to 10.4 mm in diameter ($n = 142$). For seed production, capitulum diameter thus appears to be the strongest plant-based predictor of tolerance/intolerance. We speculate that larger capitula are able to invest more resources in costly defenses (resins). Alternatively, caterpillars begin feeding in the center of the head and can possibly reach a critical developmental stage by eating only disk florets (staminate) and exiting the head prior to reaching the female ray florets on the periphery of the head.

Either way, selection for larger capitula could improve yields by increasing the number of female florets per head and improving tolerance to moth infestation. We are interested in breeding for tolerance as one strategy to minimizing dependence on synthetic pesticides, especially given the attraction of pollinators to silflower reproductive structures [24].

While the response of large and small heads was consistent between plants, for medium-sized heads, the seven plants clustered into two groups with different seed survival responses ($p < 0.001$, Figure 5, node 4). Earlier heads had more surviving seeds only in the case of medium-sized heads in three out of the seven plants (Figure 5, node 5). Head position was not identified as one of the most significant factors in percent seed survival. These results suggest that there are plant traits beyond head size that could influence the ability of seeds to escape caterpillar predation. In future experiments designed to estimate additive genetic effects, the results presented here suggest that we should focus on medium-sized heads to have the best chances of observing variation for tolerance independent of head diameter.

Because insects could quickly evolve to overcome plant defenses or mechanisms providing insect damage tolerance, agronomic and ecological research designed to limit eucosma populations will be required to enable breeding to keep pace with pest evolution in the long term. Strategies that deserve attention include use of synthetic sex attractants [33,34], biological control products [35], and strategic intercropping or habitat management to foster populations of natural enemies and parasitoids [36].

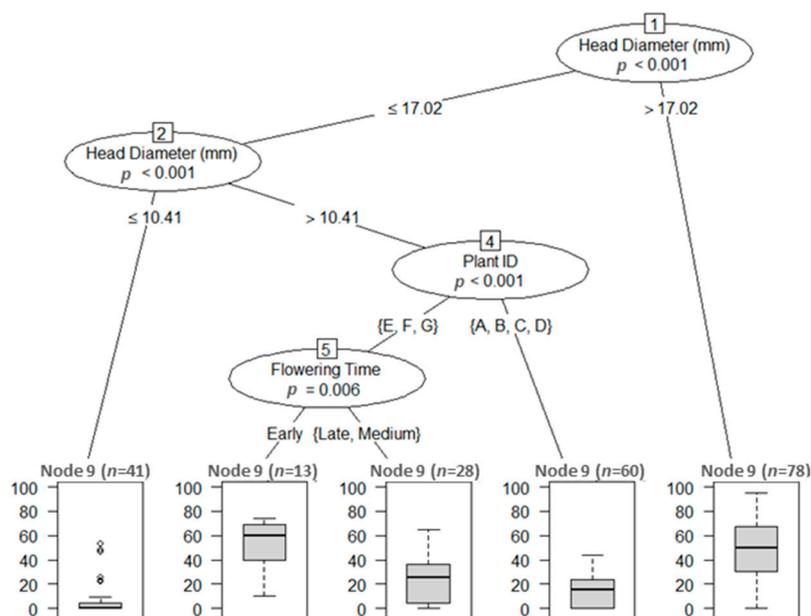


Figure 5. Conditional inference tree for 218 *Silphium integrifolium* capitula containing *Eucosma giganteana* larvae. Response variable: percent of achenes escaping predation. Candidate predictors (ovals) included capitulum and receptacle diameters, plant identity (ID) capitulum position and flowering time. For each inner node, the node is labeled according to the predictor responsible for the split and the Bonferroni-adjusted p -values are given. A boxplot (rectangles) presenting percent of escaped seeds per head is displayed for each terminal node.

3.2. Progress in Crop Management

3.2.1. Propagation

Silflower can be established as a crop by direct seeding or planting out seedlings with four true leaves. Seedlings can be produced in germination trays from stratified seeds (see Section 2) and transplanted to the field 45 days after seeding, either in late spring or fall. We report here anecdotal results derived from many attempts to establish silflower plots.

Spring transplanting: Plants remain as vegetative rosettes during the 12 months following transplantation (typically December in Patagonia; May in Kansas). Excellent survival is common, even when seedlings are set in the field in the form of small plugs (3.9 cm², 5.7 cm deep) without irrigation except spot irrigation during the transplanting process. However, at the end of spring (e.g., late June or early July in Kansas) seedling survival can be very poor if there is hot weather immediately after transplanting. No ill effects of transplanting into cold soil have been noted and, in general, silflower seedlings tolerate cold and even frosts very well. **Autumn transplanting:** we have had success transplanting until late December in Kansas. Seedlings transplanted in fall will die back during the winter and a minority of plants will bolt and flower the next spring. The majority of plants will remain vegetative until the following spring, but will produce very robust rosettes during the first spring and summer. Losses during the winter appear to be caused more by excessive or inadequate moisture in the top 10 cm of the soil than by low temperature.

Direct seeding: Seeds mechanically planted or drilled approximately 1–2 cm can germinate and emerge well. However, silflower seed is strongly dormant until after 3–6 weeks of cold, moist conditions. In the laboratory, seed can be stratified and quickly re-dried as a form of seed “priming” that allows the dry seed to be planted. However, in the field, planting dormant or primed seed has often failed in the spring and summer, suggesting that warm soil reverses the dormancy-breaking effects of stratification. These plantings typically germinate the next spring.

We report success from planting seed in late autumn or early winter to allow natural stratification during the winter and early spring. The optimal temperature for seed germination is not known. Moist, stratified silflower seed frequently germinates in a refrigerator (4 °C) but also in germination chambers/greenhouses 24–29 °C.

Vegetative propagation is an option to reproduce desired phenotypes, either by stem cuttings (see Section 2) or by splitting the crown, although the latter is slower and more laborious because of the woody nature of the crown. Crowns can be dug and divided any time between vegetative senescence and bolting the next spring. Dormant buds are very noticeable and at least one should be included with each piece of rhizome. Rhizome pieces should be kept damp but do not need protection from freezing prior to transplanting.

Stem cuttings: Cutting position in the stem was the main factor affecting rooting success; independently of rooting media or the concentration of plant regulator (0, 0.1%, 0.8%, or 1.6% IBA), none of the sub-apical cuttings produced roots. Failure in rooting of sub-apical cuttings was also reported for other members of the Asteraceae [37]. We found no significant difference in rooting success between potting mix and soil:potting mix (Table 1; $F = 0.76$; $p = 0.39$). None of the cuttings placed in water beakers produced roots. Indole-3 butyric acid (IBA) source (Hormex, rooting powder, Brooker Chemical, Hollywood CA), 0.8%, was more efficient in promoting root formation than IBA 0.1% or control (Table 1; $F = 12.39$; $p < 0.01$) and the roots were thicker [38].

Table 1. Proportion of apical cuttings that developed roots 25 days after the initiation of treatments. Treatment means and standard deviations are presented.

Plant Regulator	Water	Potting Mix	Soil
Control	0%	46.6 ± 6.6%	33.4 ± 6.6%
IBA 0.1%	0%	13.4 ± 6.6%	26.6 ± 6.6%
IBA 0.8%	0%	86.6 ± 6.6%	53.4 ± 13.5%

In some subsequent attempts to use this protocol, we have had near complete failure due to unknown pathogens that appear to be transmitted between cuttings by irrigation water. Future methods for vegetative cloning should focus on rooting media with excellent water drainage or even aeroponic systems.

3.2.2. Fertilization

Experiments to optimize crop nutrition frequently experimentally manipulate the levels, frequency of application, and forms of several macro-nutrients. Results are likely to be primarily applicable to particular soils and climates. Such work with silflower is being done at the University of Minnesota [39]. Our purpose here was quite different. Following nitrogen fertilization of a plot in 2015, we observed increases in biomass but also changes in flowering and branching patterns leading to reduced seed production. We wondered if a long evolutionary history of growing in nitrogen-limited grasslands had resulted in maladaptation to agricultural levels of fertility. However, in those cases no unfertilized control was available, so in 2017 we fertilized half of a large stand of silflower and did not fertilize the other half. The effect of the added nitrogen was visible on plant color and size, but we wanted to see if there were predictable developmental differences. So, individual plants were studied.

As expected, fertilization increased total leaf area, total biomass, plant height, and stalk diameter, but decreased harvest index (Table 2). However, contrary to our previous observations, seed yield also increased. Achene weight and number of achenes per head was unaffected by N addition and the number of heads per plant was the most important component affecting yields ($F = 12.43$; $p = 0.02$; $R^2 = 0.44$).

Not surprisingly, N addition was associated with increased nitrogen concentration of green leaves, increased photosynthetic rate, and decreased proportion of senescent leaves. An interesting result was that the number of lateral roots decreased in fertilized plants, suggesting that external N supply might

change internal N cycling. Altered N cycling could indirectly contribute to increased seed production because a consequence of decreased demand for N resorption is delayed leaf senescence, which, in turn, could increase current C gain through both elevated photosynthesis per unit leaf area and greater leaf area. Another important consideration that deserves additional investigation is the potential of fertilization to increase the C and N stores in the crown, contributing to greater plant longevity.

Our observation that improved nitrogen fertility does not affect seed size is important information for breeders who are trying to select for increased seed size. If this trait was highly sensitive to nitrogen availability, progress would be slowed by patchiness in nitrogen availability within breeding nurseries. This observation is also surprising because in annual sunflower seed weight was the main determinant of changes in yield with fertilization [40,41]. However, we should note experimental differences that could explain some or all of the different response to fertilization: the rate of fertilization and the timing of application differed between sunflower and silflower. On the one hand, the rate was lower in silflower (50 vs. 160 kg·ha⁻¹ [40]); on the other, urea was applied earlier in the season (vegetative rosette vs. pre-anthesis). The difference in timing may be particularly relevant because Hocking and Steer [42] proposed that final seed size is particularly sensitive to plant N status between the end of floret initiation and anthesis.

Future silflower studies should consider the addition of a second application of urea between bud appearance and anthesis. In the meantime, we are encouraged to find that silflower can respond to favorable nitrogen conditions by increasing seed production. However, the decrease in harvest index induced by additional nitrogen is a serious impediment to using nitrogen for increasing seed production because it is associated with increased height and stalk diameter. Tall, stalky plants are more difficult to harvest mechanically, requiring much more material to pass through the harvester and therefore forcing reduced speed and greater energy consumption. Tall plants are also prone to lodging, which can result in heads below the harvested height and an increase in rot and mold. Genetic dwarfing has not yet been seen in *S. integrifolium*, although some candidates have been seen in *S. perfoliatum* [16]. Plants with stalks removed in the spring prior to flowering regrew and produced an equivalent number of heads and seeds to uncut plants, but stems never got as tall or thick [43]. This preliminary finding suggests a promising method for making silflower more amenable to mechanical harvesting, although additional research will be required to study the effects on annual harvest index and nitrogen use efficiency, and to identify the value of spring harvested biomass as fodder.

Table 2. Comparison of morphological and yield-related traits of fertilized (N1) and non-fertilized (N0) silflower harvested at maturity, three months after urea application. Different letters indicate significant differences between treatments: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

TRAITS	F-Test	Treatment	
		Control	Fertilized
Plant height (m)	7.54 **	1.02 ± 0.08	1.44 ± 0.03
Total biomass (g)	31.28 ***	153.19 ± 12.2	276.25 ± 18.26
Number of stalks	2.15 ns	4.4 ± 0.6	5.8 ± 0.7
Stalk diameter (cm)	43.5 ***	0.95 ± 0.03	1.48 ± 0.07
Total leaf area (cm ²)	8.87 **	69.93 ± 5.38	100.14 ± 8.60
[N] green leaves (mg·g ⁻¹)	13.3 **	10.22 ± 0.39	25.68 ± 1.68
A (μmoles CO ₂ cm ⁻² ·s ⁻¹)	5.8 *	24.7 ± 1.03	28.60 ± 0.93
[N] crown (mg·g ⁻¹)	10.1 **	14.98 ± 0.93	28.2 ± 2.13
Leaf senescence (%)	8.39 *	18.16 ± 4.18	10.30 ± 3.10
Number of lateral roots	11.26 ***	30.8 ± 2.86	19.50 ± 1.80
Seed yield (g)	8.64 **	31.13 ± 3.32	41.44 ± 4.41
Harvest index (%)	13.5 *	23 ± 2	14 ± 3
Number of heads per plant	23.83 ***	30.40 ± 3.14	51.20 ± 2.88
Number of seeds per head	0.35 ns	40.15 ± 3.26	42.75 ± 2.97
Seed weight (g)	0.64 ns	0.023 ± 0.0015	0.021 ± 0.0012

3.3. Progress in Domestication

We designed common garden experiments with both unselected, wild populations and populations derived from The Land Institute's breeding program. These experimental plots tested whether progress has been made in the traits under direct selection, especially average achene mass and number of achenes per head. We also compared wild and selected populations for other traits that may have begun to change during the domestication process.

3.3.1. Unintentional Selection for Seedling Vigor

Poor crop establishment is one of the major constraints in the direct-seeded system and particularly in adverse growing environments [44,45]. Early vigor is a complex trait having morphological, phenological, and physiological components, such as relative growth rate, photosynthetic capacity, dry matter partitioning to leaves, and leaf area development [46,47]. For water-limited environments or in systems without irrigation after transplanting, both early vigor and drought tolerance are crucial, and it is important to find trait combinations that enable both as well as minimize tradeoffs. Drought tolerance is related to root morphology traits such as root depth and root length [48], allowing seedlings to acquire soil resources.

Most studies on early vigor are related to growth, focusing on the leaf morphological traits, and usually did not consider the root morphology of seedlings. Here, we compared the above- and below-ground mass of seedlings that had been measured non-destructively at 60 days and destructively at 135 days after germination.

Seedlings from selected accessions accumulated biomass faster than the wild ones ($37 \pm 9 \text{ mg} \cdot \text{g}^{-1} \cdot \text{day}^{-1}$ vs. $27 \pm 3 \text{ mg} \cdot \text{g}^{-1} \cdot \text{day}^{-1}$). This higher rate of growth determined that selected seedlings were larger at the time of transplanting ($0.54 \pm 0.07 \text{ g}$ vs. $0.28 \pm 0.03 \text{ g}$). Early vigor was related to changes in the allocation pattern to above- and below-ground biomass. Relative growth rate (RGR) was positively related to the shoot: root ratio (Figure 6A) and total photosynthetic leaf area (Figure 6B), and negatively related to total root length (Figure 6C).

Is this response to selection desirable or undesirable? Early vigor and related traits such as fast development of leaf area, high pre-anthesis CO_2 assimilation rates, and high relative growth rate [49] are characteristics that have been suggested as relevant in the development of high-yield, drought-tolerant crops for Mediterranean environments. However, trade-offs between traits related to early vigor and those related to root acquisition capacity have been suggested [50], and our results confirm this. Selection resulted in seedlings with superior vigor but less exploratory capacity of the roots, which might result in low drought tolerance after transplanting.

It will be worthwhile to study the root systems of mature plants to find out if reduced allocation to roots is a temporary or permanent consequence of breeding. Although RGR and root/shoot ratios were never purposefully selected for or against at The Land Institute, there may have been unconscious preference for vigorous seedlings during plot establishment. Furthermore, when cycles of sexual propagation happen as soon as most plants flower (generally in the second growing season for silflower), if slow seedling establishment could result in weak flowering or even failure to flower until the third or later growing season, natural selection would also favor higher seedling RGR.

3.3.2. Yield Components

To look for evidence of changes in components of seed yield, we grew approximately 120 individuals from each of 10 populations, five wild and five with a history of selection, for two years.

Selection enlarged plant size by 30% in two-year-old plants. This increase in biomass was mainly due to an increase in stalk and reproductive biomass. The number of heads per plant decreased by 45%, but as the number of seeds per head doubled and the individual seed weight also increased, the total outcome was a higher yield per plant and a higher harvest index (Table 3).

These results are consistent with the results reported above for seedling RGR. The simplest explanation for the increased plant size of selected vs. wild plants is that several cycles of selection for reproductive traits also had the effect of increasing RGR. Deliberate selection for reproductive traits—encouragingly—was even stronger resulting in increased harvest index. Elevated RGR is potentially an undesirable consequence because it could represent a shift from a more conservative growth strategy to a more acquisitive one [51].

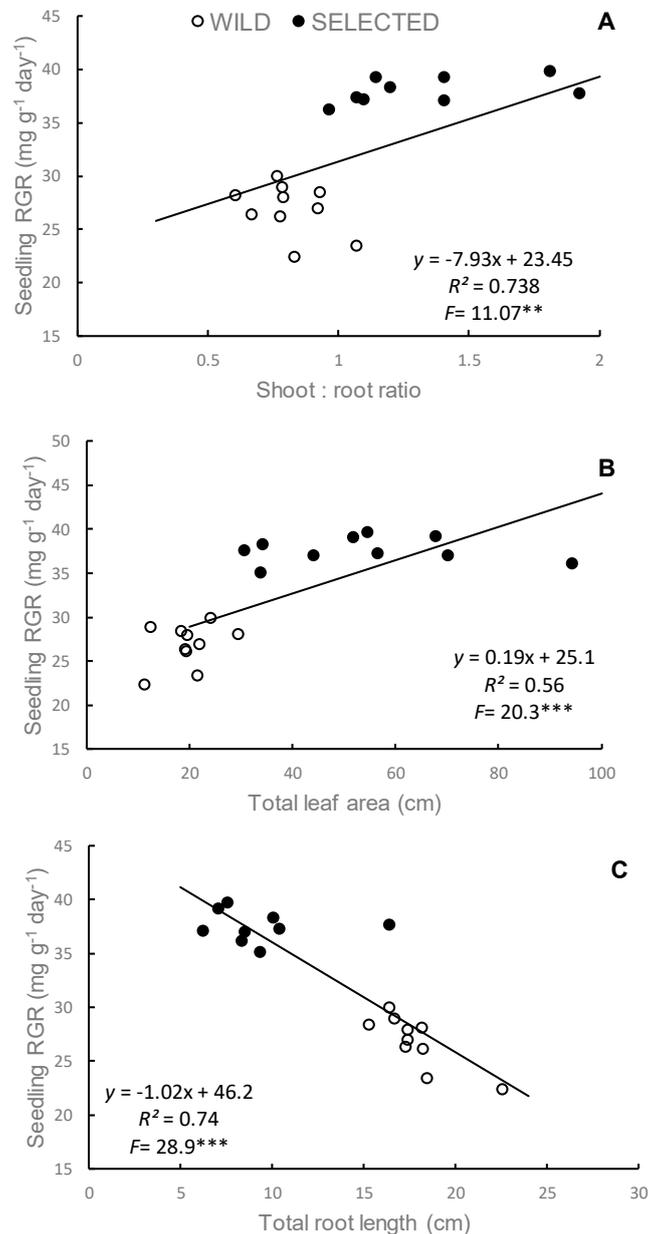


Figure 6. Relationships between seedling relative growth rate (RGR; as a proxy for early vigor) and shoot: root ratio (A); total leaf area (B); and total root length (C) for wild (open symbols) and selected (closed symbols) *Silphium* accessions. Equations and R^2 values correspond to the regression line of both accessions together.

Table 3. Comparison of morphological and yield-related traits of wild and selected silflower accessions. The means and standard deviations are presented for selected and wild treatments. DW is dry weight. Different letters indicate significant differences between treatments based on a t-test: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Trait	Selected	Wild	T-test
Total Biomass (g)	1099 ± 82.24	827.77 ± 62.90	2.49 *
Stalk Biomass (g·DW)	583.54 ± 50.54	402.23 ± 32.28	3.07 ***
Stalks: number	9.88 ± 0.66	11.32 ± 0.82	−1.38 ns
Plant height (cm)	216.38 ± 7.02	181.07 ± 7.33	3.43 **
Reproductive biomass (g·DW)	200.85 ± 20.03	136.66 ± 11.45	2.36 *
Heads (num. per plant)	168.76 ± 18.74	307.00 ± 52.78	−2.51 *
Individual seed weight (g·DW)	0.03 ± 0.0001	0.02 ± 0.0001	3.88 ***
Harvest Index	0.21 ± 0.01	0.15 ± 0.02	2.98 ***
Yield per plant (g·DW)	194.77 ± 18.43	108.24 ± 12.21	4.05 ***

3.3.3. Resin Content

Resins in plant vegetation may be involved in defense and it is important to retain them during domestication [52]; however, allocation to hydrocarbon-rich defense compounds could be compromised by strong selection for increased reproductive effort. Resins are evident in several *Silphium* species, including *S. integrifolium* [53]. We hypothesized that allocation to hydrocarbon-rich defense compounds could be compromised by strong selection for increased reproductive effort. We also hypothesized that resin production is an inducible response to disease or stress. Since rust pressure is higher in Kansas than in Patagonia, we expected to see higher levels of leaf resins in the latter and higher levels in wild than in selected populations.

The resin content of the leaves was found to be higher in wild than in selected accessions in Kansas ($p < 0.01$, Figure 7), supporting the hypothesis that selection has reduced resin content. Additionally, these same accessions, both wild and selected, had lower resin content in Patagonia—an environment in which *Silphium* rust and most of its insect pests have not been detected—than in rust-prone Kansas fields ($p < 0.01$). Altogether, these data generate circumstantial evidence that resin is somehow involved in responding to stress and possibly in pest and pathogen resistance.

Although selection for high seed yield within a crop's native range would normally be expected to maintain plant defenses against common and severe enemies, in this case disease and insect pressures were low in the early years of the selection program. Temporary release from predation and strong selection for allocation to seeds could have resulted in reduced allocation to defense chemicals.

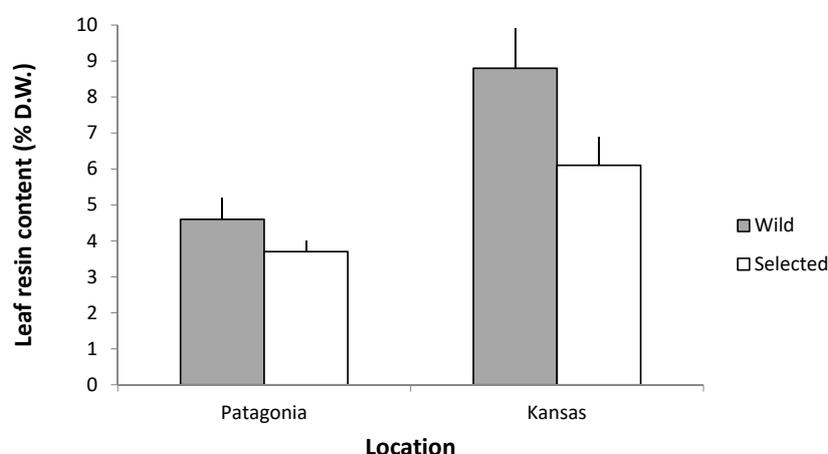


Figure 7. Resin content (DCM extracts) for leaves of wild and selected accessions grown in Kansas (USA) and in Patagonia (Argentina) Resin is expressed as % over dry weight basis. Error bars represent the mean ± S.E.

3.4. Heritability of Traits

A summary of the phenotypic data from 10 years of breeding is presented in Table S2. The data, derived from observations on individual, spaced plants, have served to inform selection of parents for breeding. They may have some value as a benchmark for future comparison, but we have not attempted to directly compare these breeding populations with each other because the populations were grown in different years and fields, few traits were measured in all populations, and there were differences in the date and method of measuring traits.

Moderate to high heritability estimates have been made at one time or another for almost every trait studied (Table S2). We highlight a few important traits here. Ligule number, which corresponds to the number of fruiting, female-fertile ray florets: 0.78. This high value is likely the result of rare “feminization alleles” accumulating to intermediate frequency as the result of several cycles of recurrent selection between 2003 and 2010. Achene mass: 0.21–0.59. Increasing seed size is an important goal for most new crops in part because it is a component of seed yield but also because larger seeds are easier to plant and clean. This is a particular priority for silflower because the achenes are flat and catch the wind, making seed cleaning especially difficult. Differences in how heads are subsampled or how thoroughly samples are cleaned could explain some of the variation in heritability estimates for this trait. Leaf health/leaf shape/rust resistance: 0.24–0.70. We are optimistic about the prospects for developing new breeding populations with durable and effective resistance to rust and other as-yet-undiagnosed conditions that can cause leaf shape abnormality. Visual scoring of leaf health has proven to be effective and has the advantage of being rapid and inexpensive. Lodging/erectness: 0.18–0.41. Even low–moderate heritability for these scores is encouraging because these are rapid, visual, holistic assessments that require mental averaging across stalks with different degrees and types of “lodging” (damaged vs. curved/twisted vs. straight but leaning) or the inverse (“erectness”). We initially expected that heritable differences for lodging would be difficult to find due to environmental “noise” (localized drainage issues, wind eddies, animal traffic). Our current hypothesis is that most lodging is caused by damage to stems and crowns by stem-boring insects whose populations have become high enough that almost no stalks escape them by chance alone.

4. Discussion

4.1. Crop Biology and Ecology

Stress tolerance, ecosystem services: Silflower acquires water and nitrogen from up to 2 m deep, which could allow it to escape temporary droughts and serve in a bioremediation role. To more fully model the environments in which this new crop may have the greatest comparative advantage, we require more information about the water use efficiency, rooting depth, and water uptake in different kinds of soils, and response to water deficits (drought tolerance vs. avoidance).

Crop health *Puccinia* and *Eucosma* resistant/tolerant silflower varieties are a prerequisite for farmer adoption in North America. Specialist pests and diseases endemic to the prairie/plains region of North America can nearly eliminate seed production when populations build up in a local area. Rust seriously impairs plant source apparatus and the giant eucosma moth can destroy seeds, stems, and rhizome sinks. Additional work is needed to culture the rust (*P. silphii*) in vitro to develop seedling inoculation techniques. The level of diversity of *P. silphii* and its propensity to reproduce sexually are areas of additional interest due to their application in determining the number of resistance genes needed for durable protection. The level of diversity of *P. silphii* and its propensity to reproduce sexually are areas of additional interest to ensure durable protection through genetic resistance. As this new species is domesticated, it will be crucial to maintain diversity for resistance genes as well as to determine how growing *Silphium* species in agricultural settings will influence rust infection levels. We intend to breed aggressively for increased giant eucosma moth (*Eucosma giganteana*) tolerance, both by continuing to select for larger capitula and also by directly selecting for seed survival in infested heads. The results presented above suggest that subsampling of a few capitula, out of the

hundreds on each plant, could efficiently indicate a plant's ability to tolerate eucosma caterpillars if those heads were chosen from early flowering heads in the medium head diameter class.

Yield potential: Potential yield per plant is high, with an average of 194 g of achenes per plant in Patagonia (Table 3). For context, Australian and American cultivars of annual sunflower average about 50 g of achenes per plant. High yields of silphium can be obtained outside the native range of distribution of *Silphium integrifolium* (e.g., Patagonia, Minnesota [39]), where pathogens and pests do not currently affect the plants. Yield per hectare is reduced by silflower's remaining wild traits, including the indeterminate production of heads and seed shattering. Increased yield per hectare will also require crop management research to identify optimal plant density. New types of management, including biomass harvesting before or after flowering, could increase optimal plant density. Plant breeding can also increase optimal density by reducing intra-specific competition (e.g., reduced plant height, branching), or increasing tolerance to crowding. The year-long juvenile, rosette stage of silflower may be necessary for deep root development, although this establishment year clearly reduces silflower annual average yield per hectare. Developing forage or grain "nurse crops" for the establishment year may be important for making silflower economically viable for farmers.

4.2. Crop Management

Propagation: Silflower can be propagated sexually and asexually. Protocols for research-scale propagation are provided, but additional research is needed to optimize propagation for production scale. Direct seeding in the field is currently only recommended when seed is planted in the cool season to take advantage of natural stratification.

Nitrogen response: Silflower seed yield responds to increased N availability but biomass yield responds more, resulting in reduced harvest index. N supply at rosette stage ($50 \text{ kg}\cdot\text{ha}^{-1}$) increases 30% seed yield mainly as a consequence of an increase in the number of heads per plant and an 80% increase in biomass production. Fertilized plants improve both in C balance by the increase in [N] in green leaves and photosynthetic rate, and in N use efficiency by a reduction in leaf senescence. In future research to improve harvest index, we need to consider a higher rate of N supply and split the doses of N applied in two moments in the growth cycle: (1) early in the vegetative stage to increase vegetative growth and increase N use efficiency and C gain; and (2) between bud appearance and anthesis to increase seed set and seed growth.

4.3. Crop Domestication

Unintended consequences. Selection appears to increase the relative growth rate (early vigor and plant biomass), reducing drought tolerance (root length) and defense (resin content). We have begun to monitor traits that were not under direct selection in order to determine if undesirable changes are beginning. For example, imbalance between the transpiring leaf area and the absorptive root surface could compromise seedling survival in water-limited agricultural systems. Additional research is required to weigh the costs and benefits of changes like this before corrections can be made to breeding criteria.

Intended consequences. Cycles of selection for domestication traits have increased the number of achenes per head and the size of achenes. As a consequence, harvest index has also modestly increased.

Future priorities. Achieving a domesticated plant form has become a top priority for production outside of silflower's native range. Within the native range, insect and disease tolerance/resistance are the top priority. As is true for annual crops, we anticipate that domesticated silflower will need more synchronized flowering, reduced shattering, and reduction of plant size. These goals can partially be achieved through the development of management practices such as pre-flowering cut of apical portions of stems to reduce plant stature and homogenize the height at which heads are produced, but, at a minimum, we anticipate the need to breed for improved plant response to these management regimes.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2071-1050/10/3/638/s1>, Table S1: *Silphium integrifolium* accessions acquired by The Land Institute, 1999–2013, Table S2: Traits measured in breeding nurseries at The Land Institute, 2007–2017, Table S3: Trait definitions for *Silphium integrifolium* breeding.

Acknowledgments: Field and lab work in Argentina was partially funded by PIP 2015–2017 (Damian Ravetta). The Perennial Agriculture Project, a joint project between The Land Institute and The Malone Family Land Preservation Foundation, provided partial support for work conducted in Kansas. Sheila Cox, Laura Kemp, John Holmquist, James Bowden, and Dimas Teran were technicians on these projects. Many supporters of The Land Institute collected seeds from wild *Silphium* populations and donated them to us. *Helianthus* seeds were provided by the Germplasm Resources Information Network of the USDA. USDA entomologist Jarrad Prasifka identified *Eucosma giganteana* and other pests in our fields and taught us how to identify them at various stages. AgriFood Canada plant pathologist Sarah Hambleton helped identify and sequence our samples of *Puccinia silphii*.

Author Contributions: Alejandra Vilela, Luciana González-Paleo, and Damian Ravetta conceived, designed, and performed the experiments in Patagonia. Kathryn Turner and Damian Ravetta performed the rust and resin experiments. Kelsey Peterson performed the insect experiments. David Van Tassel collected/acquired wild *Silphium* germplasm and developed the selected populations. Timothy Crews designed and analyzed the ¹⁵N and leaf water potential experiments. Each author wrote his/her section of the manuscript. The paper was compiled and edited by David Van Tassel and Alejandra Vilela.

Conflicts of Interest: The authors declare no conflict of interest.

References

- Glover, J.D.; Reganold, J.P.; Bell, L.W.; Borevitz, J.; Brummer, E.C.; Buckler, E.S.; Cox, C.M.; Cox, T.S.; Crews, T.E.; Culman, S.W.; et al. Increased food and ecosystem security via perennial grains. *Science* **2010**, *328*, 1638–1639. [[CrossRef](#)] [[PubMed](#)]
- Cox, T.; Glover, J.; Tassel, D. Van Prospects for developing perennial grain crops. *Bioscience* **2006**, *56*, 649–659. [[CrossRef](#)]
- Crews, T.E.; Blesh, J.; Culman, S.W.; Hayes, R.C.; Jensen, E.S.; Mack, M.C.; Peoples, M.B.; Schipanski, M.E. Going where no grains have gone before: From early to mid-succession. *Agric. Ecosyst. Environ.* **2016**, *223*, 223–238. [[CrossRef](#)]
- Sanderson, M.A.; Adler, P.R. Perennial forages as second generation bioenergy crops. *Int. J. Mol. Sci.* **2008**, *9*, 768–788. [[CrossRef](#)] [[PubMed](#)]
- Cadisich, G.; Row, E.; van Noordwijk, M. Nutrient harvesting—The tree-root safety net. *Agrofor. Forum* **1997**, *8*, 31–33.
- Bhat, R.; Karim, A.A. Exploring the Nutritional Potential of Wild and Underutilized Legumes. *Compr. Rev. Food Sci. Food Saf.* **2009**, *8*, 305–331. [[CrossRef](#)]
- Lefroy, E.C.; Flugge, F.; Avery, A.; Hume, I. Potential of current perennial plant-based farming systems to deliver salinity management outcomes and improve prospects for native biodiversity: A review. *Aust. J. Exp. Agric.* **2005**, *45*, 1357–1367. [[CrossRef](#)]
- Singh, A.; Krause, P.; Panda, S.N.; Flugel, W.-A. Rising water table: A threat to sustainable agriculture in an irrigated semi-arid region of Haryana, India. *Agric. Water Manag.* **2010**, *97*, 1443–1451. [[CrossRef](#)]
- Van Tassel, D.L.; Albrecht, K.A.; Bever, J.D.; Boe, A.A.; Brandvain, Y.; Crews, T.E.; Gansberger, M.; Gerstberger, P.; González-Paleo, L.; Hulke, B.S.; et al. Accelerating silphium domestication: An opportunity to develop new crop ideotypes and breeding strategies informed by multiple disciplines. *Crop Sci.* **2017**, *57*, 1274–1284. [[CrossRef](#)]
- Weaver, J.E.; Stoddart, L.A.; Noll, W. Response of the Prairie to the Great Drought of 1934. *Ecology* **1935**, *16*, 612–629. [[CrossRef](#)]
- United States Department of Agriculture, Natural Resources Conservation Service. The PLANTS Database. Available online: <http://plants.usda.gov> (accessed on 17 September 2016).
- Cox, S.; Nabukalu, P.; Paterson, A.; Kong, W.; Nakasagga, S. Development of Perennial Grain Sorghum. *Sustainability* **2018**, *10*, 172. [[CrossRef](#)]
- Vail, J.; Kulakow, P.; Benson, L. Illinois Bundleflower: Prospects For A Perennial Seed Crop. In Proceedings of the Twelfth North American Prairie Conference, Cedar Rapids, IA, USA, 4–6 August 1980; pp. 31–32.
- Jackson, W. Nature as the measure for agriculture. In *Ecology, Economics, Ethics: The Broken Circle*; Borkmann, F.H., Kellert, S.R., Eds.; Yale University Press: New York, NY, USA; London, UK, 1991; pp. 43–58.
- Jackson, W. *New Roots for Agriculture*; University of Nebraska Press: Lincoln, NE, USA, 1985.

16. Van Tassel, D.L. (The Land Institute, Salina, KS, USA). Personal communication, 2017.
17. Boyer, J.S. *Measuring the Water Status of Plants and Soils*; Academic Press: Cambridge, MA, USA, 1995.
18. Hothorn, T.; Hornik, K.; Zeileis, A. Unbiased Recursive Partitioning: A Conditional Inference Framework. *J. Comput. Graph. Stat.* **2006**, *15*, 651–674. [[CrossRef](#)]
19. R_Core_Team R: A Language and Environment for Statistical Computing. Available online: <http://www.r-project.org/> (accessed on 1 December 2017).
20. Strobl, C.; Malley, J.; Tutz, G. An introduction to recursive partitioning: Rationale, application, and characteristics of classification and regression trees, bagging, and random forests. *Psychol. Methods* **2009**, *14*, 323–348. [[CrossRef](#)] [[PubMed](#)]
21. Green, H.; Curtis, J. Germination studies of Wisconsin prairie plants. *Am. Midll. Nat.* **1950**, *43*, 186–194. [[CrossRef](#)]
22. Hunt, R.; Causton, D.R.; Shipley, B.; Askew, A.P. A modern tool for classical plant growth analysis. *Ann. Bot.* **2002**, *90*, 485–488. [[CrossRef](#)] [[PubMed](#)]
23. Kristensen, H.L.; Thorup-Kristensen, K. Root Growth and Nitrate Uptake of Three Different Catch Crops in Deep Soil Layers. *Soil Sci. Soc. Am. J.* **2004**, *68*, 529–537. [[CrossRef](#)]
24. Prasifka, J.R.; Mallinger, R.E.; Hulke, B.S.; Larson, S.R.; Van Tassel, D. Plant–Herbivore and Plant–Pollinator Interactions of the Developing Perennial Oilseed Crop, Silphium integrifolium. *Environ. Entomol.* **2017**. [[CrossRef](#)] [[PubMed](#)]
25. Horst, R. *Westcott's Plant Disease Handbook*; Springer: New York, NY, USA, 2008.
26. Wagner, A.; Jamiolkowska, A. First Report of *Alternaria alternata* Causing Stem Blight of Compass Plant (*Silphium laciniatum*) in Poland. *Plant Dis.* **2004**, *88*, 1045. [[CrossRef](#)]
27. Siddiqui, M.; Brown, J. Effects of simulated rust epidemics on the growth and yield of sunflower. *Aust. J. Agric. Res.* **1977**, *28*, 389. [[CrossRef](#)]
28. Markell, S.; Gulya, T.; McKay, K.; Hutter, M.; Hollingsworth, C.; Ulstad, V.; Koch, R.; Knudsvig, A. Widespread Occurrence of the Aecial Stage of Sunflower Rust Caused by *Puccinia helianthi* in North Dakota and Minnesota in 2008. *Plant Dis.* **2009**, *93*, 668. [[CrossRef](#)]
29. Arthur, J.C. *Manual of the Rusts in United States and Canada*; Purdue Research Foundation: Lafayette, IN, USA, 1934.
30. Parmelee, J.A. The Autoecious species of *Puccinia* on Heliantheae in North America. *Can. J. Bot.* **1967**, *45*, 2267–2327. [[CrossRef](#)]
31. Hambleton, S. (Agriculture and Agri-Food Canada, Ottawa, ON, Canada). Personal communication, 2017.
32. Prasifka, J.R. (United States Department of Agriculture, Fargo, ND, USA). Personal communication, 2017.
33. Sartwell, C.; Daterman, G.E.; Koerber, T.W.; Stevens, R.E.; Sower, L.L. Distribution and Hosts of *Eucosma sonomana* in the Western United States as Determined by Trapping with Synthetic Sex Attractants. *Ann. Entomol. Soc. Am.* **1980**, *73*, 254–256. [[CrossRef](#)]
34. Rogers, C.E.; Thompson, T.E.; Jones, O.R. *Eucosma womonana* Kearfott (*Lepidoptera: Olethreutidae*): A New Pest of Sunflower in the Southern Plains. *J. Kansas Entomol. Soc.* **1979**, *52*, 373–376.
35. Lacey, L.; Georgis, R. Entomophagenic nematodes for control of insect pests above and below ground with comments on commercial production. *J. Nematol.* **2012**, *44*, 218–225. [[PubMed](#)]
36. Fiedler, A.K.; Landis, D.A.; Wratten, S.D. Maximizing ecosystem services from conservation biological control: The role of habitat management. *Biol. Control* **2008**, *45*, 254–271. [[CrossRef](#)]
37. Wassner, D.; Ravetta, D. Vegetative propagation of *Grindelia chiloensis* (*Asteraceae*). *Ind. Crops Prod.* **2000**. [[CrossRef](#)]
38. Vilela, A.E. (MEF-CONICET, Trelew, Patagonia Argentina). Personal communication, 2016.
39. Schiffner, S.; Sheaffer, C. (University of Minnesota, Minneapolis, MN, USA). Personal communication, 2017.
40. Özer, H.; Polat, T.; Öztürk, E. Response of irrigated sunflower (*Helianthus annuus* L.) hybrids to nitrogen fertilization: Growth, yield and yield components. *Plant Soil Environ.* **2006**, *50*, 205–211. [[CrossRef](#)]
41. Tomar, H.P.S.; Dadhwal, K.S.; Singh, H.P. Effect of irrigation, N, and P on yield and yield attributes of spring sunflower (*Helianthus annuus* L.). *Trop. Agric.* **1999**, *76*, 228–231.
42. Hocking, P.J.; Steer, B.T. Effects of timing and supply of nitrogen on nitrogen remobilization from vegetative organs and redistribution to developing seeds of sunflower. *Plant Soil* **1995**. [[CrossRef](#)]
43. González-Paleo, L. (MEF-CONICET, Trelew, Patagonia Argentina). Personal communication, 2017.

44. Singh, U.P.; Singh, Y.; Kumar, V.; Ladha, J.K. Evaluation and promotion of resource conserving tillage and crop establishment techniques in rice-wheat system in Eastern India. In *Integrated Crop and Resource Management in the Rice-Wheat System of South Asia*; International Rice Research Institute: Los Baños, Philippines, 2009; pp. 151–176.
45. Zhang, Z.-H.; Yu, S.-B.; Yu, T.; Huang, Z.; Zhu, Y.-G. Mapping quantitative trait loci (QTLs) for seedling-vigor using recombinant inbred lines of rice (*Oryza sativa* L.). *Field Crops Res.* **2005**. [[CrossRef](#)]
46. Asch, F.; Sow, A.; Dingkuhn, M. Reserve mobilization, dry matter partitioning and specific leaf area in seedlings of African rice cultivars differing in early vigor. *Field Crops Res.* **1999**, *62*, 191–202. [[CrossRef](#)]
47. Caton, B.P.; Cope, A.E.; Mortimer, M. Growth traits of diverse rice cultivars under severe competition: Implications for screening for competitiveness. *Field Crops Res.* **2003**, *83*, 157–172. [[CrossRef](#)]
48. Comas, L.H.; Becker, S.R.; Cruz, V.M.V.; Byrne, P.F.; Dierig, D.A. Root traits contributing to plant productivity under drought. *Front. Plant Sci.* **2013**, *4*, 442. [[CrossRef](#)] [[PubMed](#)]
49. López-Castañeda, C.; Richards, R.A.; Farquhar, G.D.; Williamson, R.E. Seed and seedling characteristics contributing to variation in early vigor among temperate cereals. *Crop Sci.* **1996**, *36*, 1257–1266. [[CrossRef](#)]
50. Rebolledo, M.C.; Luquet, D.; Courtois, B.; Henry, A.; Soulié, J.-C.; Rouan, L.; Dingkuhn, M. Can early vigour occur in combination with drought tolerance and efficient water use in rice genotypes? *Funct. Plant Biol.* **2013**, *40*, 582–594. [[CrossRef](#)]
51. González-Paleo, L.; Ravetta, D.A. Indirect changes associated with a selection program for increased seed-yield in wild species of *Lesquerella* (*Brassicaceae*): Are we developing a phenotype opposite to the expected ideotype? *Ind. Crops Prod.* **2011**, *34*, 1372–1380. [[CrossRef](#)]
52. McKey, D.; Elias, M. Ecological approaches to crop domestication. In *Biodiversity in Agriculture: Domestication, Evolution and Sustainability*; Gepts, P., Famula, T.R., Bettinger, R.L., Brush, S.B., Damania, A.B., Eds.; Cambridge University Press: Cambridge, MA, USA, 2012; pp. 377–406.
53. Langenheim, J.H. *Plant Resins: Chemistry, Evolution, Ecology and Ethnobotany*; Timber Press: Portland, OR, USA; Cambridge, MA, USA, 2003.



© 2018 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).