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Communication

Review of Partial Hybrids between Herbaceous *Medicago sativa* and Woody *Medicago arborea* and Their Potential Role in Alfalfa Improvement

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Simple Summary: Partial hybrids between *Medicago sativa* (alfalfa) and *Medicago arborea*, named Alborea, are the result of reproductive abnormalities in the *M. sativa* seed parent. These hybrids have the potential, through the addition of traits outside the alfalfa gene pool, to increase the productivity and utilisation of alfalfa.

Abstract: *Medicago sativa* (2n = 4x = 32) and *M. arborea* (2n = 4x = 32) were thought to be reproductively isolated until hybrids (Alborea) were produced by sexual reproduction for the first time in 2003 in Wisconsin. The hybrids were asymmetric, at or near 2n = 4x = 32, and with a predominance of the alfalfa genome. Only *M. sativa* seed parents with reproductive abnormalities, including unreduced eggs, have produced hybrids; where *M. arborea* has been used as the seed parent, no hybrids have resulted. Pedigree selection within derivatives of the two original *M. sativa* seed parents (MB and M8) has been successful in increasing the frequency of hybrids produced. While Alborea individuals more closely resemble *M. sativa*, a number of *M. arborea*-specific traits have been observed across different hybrid individuals. These include single-coil flat pods, large seeds, yellow flowers, indeterminate growth, a minimal crown, lodging, frost resistance, and anthracnose resistance. These *M. arborea* traits have the potential to restructure alfalfa to increase its versatility and utilisation. There is emerging evidence from North and South America and Australia that some Alborea selections have the capacity to complement adapted alfalfa cultivars for yield. Work is continuing to introgress *M. arborea* traits of value into alfalfa.

Keywords: partial hybrids; interspecific hybrids; unreduced gametes; reproductive abnormalities; plant breeding

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1. Introduction

Alfalfa (*Medicago sativa* L.) is the world's oldest known cultivated forage species; historical records date back to 1300 BC in Turkey [1]. Alfalfa is grown in over 80 countries, and the world area is in the range of 30–35 m ha, of which 70% is collectively located in the USA, former USSR, and Argentina [2]. Alfalfa's wide usage is driven by it possessing valuable agronomic traits, including a high feeding value, its perenniality, and its ability to fix atmospheric nitrogen [3]. Also, the deep rootedness of alfalfa makes it suitable for reducing nitrate leaching and preventing the expansion of salt-degraded soils. Cultivated alfalfa is autotetraploid (2n = 4x = 32) [4] as well as being subject to outbreeding and very subject to inbreeding depression. Because of this, alfalfa breeding methodologies leading to commercialisation as synthetic cultivars have remained largely unchanged over several decades [5]. This has contributed to a yield stagnation, which has developed in both North America [6,7] and Australia [8].

In alfalfa breeding, two subspecies have been extensively exploited; the purple-flowered *M. sativa* subspecies sativa and the yellow-flowered *M. sativa* subspecies *falcata*.

Other taxa in the above subspecies complex include *M. sativa* subspecies *glomerata*, *M. sativa* subspecies *varia*, *M. sativa* subspecies *caerulea*, and *M. sativa* subspecies × *hemicycla*. Both 2n = 4x = 32 and 2n = 2x = 16 exist, except for *M. sativa* subspecies × *hemicycla*, subspecies *caerulea*, and subspecies *glutinosa*; all of which are 2n = 2x = 16 [9]. Until now, the above subspecies within the *M. sativa* complex have provided the gene pool for alfalfa cultivar improvement. However, despite the breeding of multiple disease- and pest-resistant cultivars over the last 100 years as well as winter hardiness, alfalfa yields and persistence levels appear to have plateaued.

High levels of genetic diversity exist within the *M. sativa* subspp. Intermating between multiple alfalfa germplasms over the entire subspp. complex has been extensively practised since 1950 in an attempt to ingress multiple agronomically valuable traits [10]. However, there are traits not present in the *M. sativa* subspecies complex that would increase the utilisation of alfalfa; these include a larger seed size, increased drought tolerance, and increased tolerance to salinity [11]. All of these traits and others are known to exist in *Medicago arborea*. In this paper, we review research undertaken to generate hybrids between *M. sativa* and *M. arborea*, with the aim of introgressing traits unique to *M. arborea* such as a larger seed size into the alfalfa gene pool.

2. Phylogenetic Relationships between *M. sativa* subspp. and *M. arborea*

The genus *Medicago* has received extensive revision since it was first comprehensively monographed by Urban [12], where 46 species were recognised. Small and Jomphe [13] provided a comprehensive revision of the genus based on morphology, and defined 83 species spread across 12 sections of the genus. The perennial species *M. arborea* and *M. strasseri* were placed in section *Dendrotelis* and *Medicago sativa* subspp. in section *Medicago*; the distinction is based on section *Dendrotelis* having ligneous branches and section *Medicago* having herbaceous to lignescent branches. Within *Dendrotelis*, three species have been recognized; *M. arborea*, *M. strasseri* (both 2n = 4x = 32), and *M. citrina* (2n = 6x = 48). The main distinction between *M. arborea* and *M. strasseri* was the fruit size, with the fruit and seed diameter twice as large in *M. arborea*.

Molecular Phylogenies

Steele et al. [14] used plastid and nuclear DNA sequences to reassess phylogeny and character evolution in *Medicago*. *Medicago sativa* subspp. and relatives in section *Medicago* formed a weakly to strongly supported monophyletic group, which was referred to as the section *Medicago* clade [14]. *Medicago arborea* and its close relatives (previously placed in section *Dendrotelis*) always resolved within the *Medicago* clade. Steele et al. [14], based on an analysis of all molecular markers employed, concluded that *M. arborea* was part of a group with other species of section *Medicago*, although it often resolved as part of a basal polytomy within that group. They also proposed that the common ancestor of these shrubby polyploid species was a herbaceous perennial in section *Medicago*; woodiness is a derived character state of these species. It was also concluded that hybridisation is difficult to accomplish between species in section *Medicago.*

3. Early Attempts to Hybridise *M. sativa* and *M. arborea*

Several previous attempts have been made to generate hybrids between *M. sativa* and *Medicago* spp. outside the *M. sativa* subspecies complex using sexual reproductive processes [15,16]. In the work of Fridriksson and Bolton [15], pollination of *M. sativa* stigmas from highly self-incompatible clones with a range of annual and perennial *Medicago* spp., including *M. arborea*, failed to produce mature embryos. However, following pollination with *M. arborea*, the early stages of embryonic growth were initiated, indicating fertilisation had taken place. Interspecific hybrids were obtained by crossing 2x *M. sativa* with a range of diploid perennial *Medicago* spp., including *M. papillosa* [17]. Ovule embryo culture was necessary for the recovery of hybrids with balanced genomes of the two species. Only tetraploid hybrids were fertile in *M. sativa* × *M. papillosa*. These showed

only disomic segregation patterns, indicating little or no exchange between the homologous genomes [18].

Somatic hybrids were generated between *M. sativa* and *M. arborea* through the symmetrical fusion of mesophyll protoplasts of *M. sativa* with callus protoplasts of *M. arborea* [11]. While considerable genomic rearrangements were evident in the resulting hybrids, their morphology was generally intermediate to the parents, and about half of the species-specific RFLP bands of both parents was present in them. These somatic hybrids did not flower during the first 2 years after generation, even though several floral buds were observed. The authors noted that flowering may only have been delayed because *M. arborea* takes about 2 years to flower after seeding and the paper was written only 2 years after generating the hybrids. Mizukami et al. [19] used the electrofusion of protoplasts of the annual species *M. rugosa* and *M. scutellata* with 4x *M. sativa* to produce somatic hybrids. These hybrids were unstable and chromosomes were rapidly lost during vegetative growth.

4. Generation of Alborea and Its Characteristics

Alfalfa and *M. arborea* were considered to be reproductively isolated until relatively recently. McCoy and Echt [20] stated that it was possible to obtain hybrids between alfalfa and all other species of the subgenus *Medicago*, with the exception of *M. arborea*. The paper by Nenz et al. [11], where sterile somatic hybrids were reported between *M. sativa* and *M. arborea*, rekindled interest in hybrids between the two species in Wisconsin, and screening of male sterile alfalfa seed parents commenced in 1998.

Over the period 1998–2002 in Wisconsin, 5 alfalfa seed parents yielded no hybrids after pollinating at least 200 florets per plant per year. In 2003, the first hybrids were produced after pollinating clone MB (obtained from Magnum 111 X Blaser XL). Over the period 2002–2013, MB produced a hybrid for every 250 florets pollinated. A progeny of MB, WA 2071, yielded a hybrid at the rate of one for every 420 florets pollinated [21]. MB and its derivatives represented the first level of weakening the hybridisation barrier between *M. sativa* and *M. arborea*. The hybrids were described as a new cultigen, Alborea [22]. Another alfalfa genotype, M8, was discovered in Wisconsin, which was a more efficient hybridiser with *M. arborea* than MB and its derivatives [21]. M8 was derived from crossing *M. sativa* subspecies *sativa*, *caerulea*, and *falcata* in a three-way x three-way cross. M8 produced a hybrid for every 85 florets pollinated [21]. An S1 plant of M8 tested in Queensland, WA2570, yielded a hybrid for every 38 florets pollinated [23]. Both MB and M8 produce small amounts of pollen; both have been shown to possess the reproductive abnormalities of unreduced eggs and pollen [23].

The reciprocal cross *M. arborea* × *M. sativa* has failed to produce hybrids despite pollinating hundreds of *M. arborea* florets over 5- and 4-year periods in Wisconsin and Queensland, respectively [21]. This indicates that in the *M. arborea* seed parents, the hybridisation barrier is complete as it appears to be in several alfalfa plants from commercial cultivars pollinated with *M. arborea*.

4.1. Morphological Characterization

Morphologically, the hybrids exhibit traits of both parents (Table 1 and Figure 1), with the greatest resemblance being to the *M. sativa* parent. Either purple- or cream-flowered *M. sativa* have always been used as the seed parent. The hybrids can be identified by variegated flowers, with the yellow pigment coming from *M. arborea*. Another diagnostic character of hybrids is a 1–1.5-coil flat seed pod; alfalfa parents had 3-coil round pods and *M. arborea* had a 1–1.5-coil flat pod. Other *M. arborea* traits found in some hybrids include winter activity, no crown, a branched root system, an erect growth habit, and large dark-green leaves [24]. *M. arborea* has seeds four times the size of alfalfa, and some hybrids exhibit seeds approaching the size of *M. arborea*. Complementation for forage yield has also been observed in other Alborea × alfalfa crosses [24–26]. Anthracnose (*Colletotrichum trifolii*) resistance has been transferred from *M. arborea* to Alborea [27] and used in alfalfa breeding.

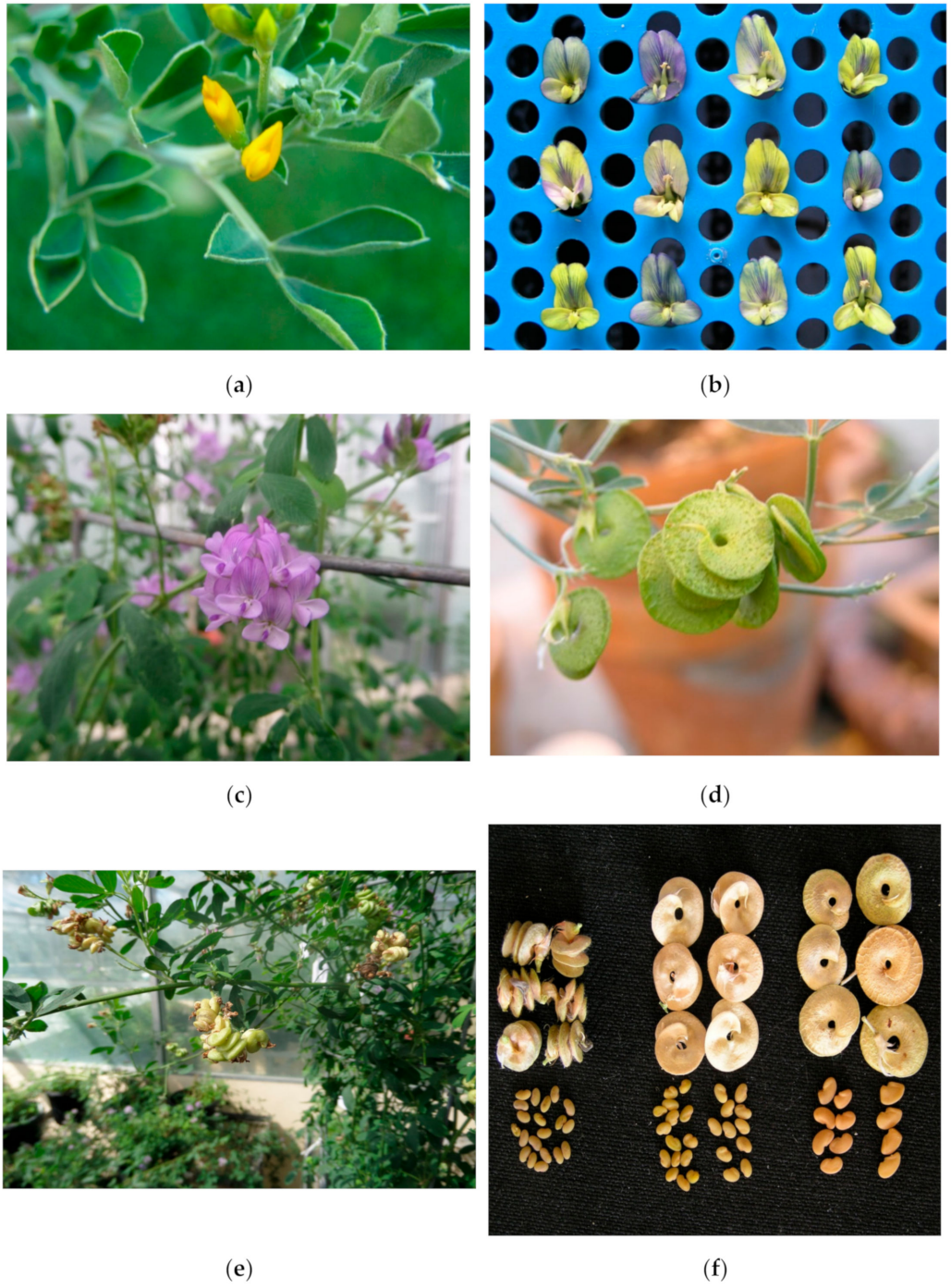

Figure 1. (**a**) Yellow/orange flowers of *M. arborea*; (**b**) co-expression of purple and yellow pigments in Alborea; (**c**) purple flower of *M. sativa* cv Sequel; (**d**) 1–1.5-coil immature flat pods of *M. arborea* ca. 1 cm in diameter; (**e**) immature 3-coil pods of *M. sativa* cv Sequel with tight coiling; (**f**) mature pods and seeds, generated in Wisconsin, of (left to right) alfalfa, Alborea, and *M. arborea.* Reproduced with permission from Ref. [24]. Copyright 2016 Australian Institute of Agricultural Science and Technology.

Table 1. *Medicago arborea* traits and other traits observed in Alborea in Wisconsin and Queensland.

Trait	Observations
Observed in greenhouses and fields	
Flower colour	Yellow flowers per se and variegated flowers
Indeterminate growth	Plants grow up to 4 m in height
Minimal crown	Observed in ca. 20% of plants
Large leaves	Leaves larger than in both parents in some plants
M. arborea pod shape and size	1- to 1.5-coil flat pods ca. 1 cm in diameter observed in ca. 20% of plants
Large seeds	Seeds twice the size of alfalfa and half the size of *M. arborea*
Short racemes	5–6 florets versus 15–25 in alfalfa
Fewer seeds per pod	0–50% of alfalfa, although 8–9 per pod observed in one plant
Pollen quantity	0–25% of alfalfa
Autogamy	Full seed set not observed; 10–25% of cross-fertility level
Crossability	Low frequency of Alborea plants that do not cross well with alfalfa but cross with Alborea
Observed in fields	
Lodging resistance	About 25% of Alborea plants resist lodging after rain and wind
Frost resistance	Low frequency of Alborea plants (ca. 5–10%) stay green down to −8 °C
Solid stem	About 5% of Alborea plants have a solid stem above the base like *M. arborea*; alfalfa has hollow stems
Heterosis in crosses	About 25% have heterosis for vigour in crosses with alfalfa
Branching roots	Some plants show absence of a tap root like *M. arborea*
Winter activity	Around 65% of plants with a group 9 dormancy level
Late flowering	May take some plants 2 years to flower, as for *M. arborea*

4.2. Genomic Characterisation

The root-tip chromosome number has been determined for Alborea and was found to be near tetraploid (2n = 4x = 30–32) [21,27]. An AFLP analysis was conducted on five hybrids and parents in Australia [27]. An MB derivative, WA2071, was used as the seed parent. The work confirmed the asymmetric genomic composition of the hybrids, with up to only 4% of the *M. arborea*-specific AFLP bands present in any hybrid. Of the bands, 27% was monomorphic in both the parents and the hybrid, so more than 4% of the *M. arborea* genome was possibly transferred. In total, 7% of the total number of bands was unique to the hybrid, indicating possible chromosomal rearrangements due to the introgression of fragments of *M. arborea* chromosome/s contributing to new alleles at the sites of introgression [27].

The presence of around 4% of *M. arborea*-specific alleles in the hybrid could indicate the transfer of a whole chromosome or the introgression of several smaller parts as, theoretically, each *M. arborea* chromosome constitutes an average of 1/32, or 3.1%, of the genome.

M. arborea introgression into 7 hybrids generated in Wisconsin [28] was investigated using the marker profiles generated by 46 SSR primers of known *M. sativa* genomic locations covering all 8 linkage groups [29–31]. Introgression of *M. arborea*-specific alleles was found from linkage groups 1, 6, and 7, with two hybrids showing introgression from all three linkage groups [32]. Test crosses were made between the hybrids and *M. sativa*, and the transmission of *M. arborea*-specific alleles was demonstrated in the test cross-progeny. Whether the introgressed *M. arborea* genome represents a chromosome, an arm, or several small pieces of chromosome/s remains unresolved, with the latter most likely.

5. Similarities between Alborea and Other Asymmetric Plant Hybrids

De Wet et al. [33] reported what they described as "counterfeit" hybrids between *Tripsacum australe* (2n = 2x = 36) and *Zea mays* (2n = 2x = 20). Hybrids were characterised by 18 or 36 *Tripsacum* and 10 or rarely 20 *Zea* chromosomes, with those containing 36 *Tripsacum* and 20 *Zea* chromosomes most likely deriving from the fertilisation of unreduced eggs and pollen. However, some hybrids resembled true hybrids in phenotype, but lacked *Zea* chromosomes and were characterised by 36 *Tripsacum* chromosomes. These individuals were named counterfeit hybrids. To explain the origin of counterfeit hybrids, it was suggested that genes had been transferred from the genome of *Zea* sperm to the genome of an unreduced egg during the fertilisation process. The situation described above is similar in many ways to what was observed with F1 Alborea hybrids. They are near tetraploid, phenotypically more closely resemble the alfalfa egg parent, but demonstrate some *M. arborea* phenotypic traits and only contain around 5% of *M. arborea*-specific alleles [27]. Unreduced eggs and pollen have been reported in Alborea parents [23,34], which were also produced in *Tripsacum* [33].

The example described above was referred to as introgressive hybridisation by Allard [35]. Here, one species is enriched to a small degree with genes derived from another, thus broadening its base of variability and increasing the variety of recombination products that may be secured from it. Allard used as an example the introgression of *Tripsacum* genes into corn by repeated natural backcrossing. As noted by Allard [35], the issue was highly speculative at that time, but there is now unequivocal evidence for introgressive hybridization [36].

Li and Heneen [37] generated intergeneric hybrids between diploid *Brassica campestris* (2n = 20) and *Orychophragmus violaceus* (2n = 24). The hybrid was mixoploid (2n = 23–42) and cells 2n = 34 were frequent. The partial separation of parental genomes during mitosis, leading to the addition of *O. violaceus* chromosomes to the *B. campestris* complement, was proposed to explain the partial hybrids.

Tu et al. [38] studied intertribal hybrids between *Brassica rapa* (2n = 2x = 20) and *Isatis indigotica* (2n = 2x = 14). One hybrid closely resembled the seed parent, *B. rapa*, and was 2n = 22. Genomic in situ hybridisation (GISH) of this F1 individual revealed two chromosomes from *I. indigotica*; the remainder were from *B. rapa*. An AFLP analysis confirmed *I. indigotica*-specific bands in the hybrid. Some of the introgressions were mitotically stable. While Alborea has not been subjected to GISH analysis, the process of formation of the partial hybrids appears to be similar to what is described above.

6. Importance of Reproductive Abnormalities in Generating Interspecific Hybrids

In the generation of Alborea, it is when only certain alfalfa genotypes are used as the seed parent that hybrids result. Repeated pollination of *M. arborea* with *M. sativa* has failed to produce hybrids [21]. It has been established that alfalfa seed parents and their derivatives that produce hybrid also produce unreduced male and female gametes [23,34]. Alfalfa parent MB had a lower frequency of 2n eggs than the other parent M8; MB is a less efficient hybridiser than M8 [34]. The production of 2n eggs, arising from second division restitution [39] by the MB or M8 parents, would appear to be integral to hybrid generation. Fertilisation of the 2n egg by a 2x gamete of *M. arborea*, followed by the subsequent loss of some *M. arborea* chromosomes due to differences in the timing of mitotic events between the two species, provides an explanation for the near 4x hybrids with a predominance of alfalfa DNA. Unreduced eggs are produced by M8 and MB at a frequency similar to that with which they produce hybrids [34]. Unreduced gamete formation in plants and their use in breeding was reviewed by Brownfield and Kohler [40]. Cheng et al. [41] reported that unreduced female gametes may have been involved in the generation of partial hybrids between *B. napus* (2n = 38) and *Orychophragmus violaceous* (2n = 24). The suspected hybrids were 2n = 38 and contained chromosome fragments of *O. violaceous*. Asynchronous mitotic cell cycles were thought to be responsible for the loss of *O. violaceous* chromosomes after fertilisation.

In the production of counterfeit hybrids between *Tripsacum* and *Zea*, it was reported that the parthenogenetic development of non-reduced female gametes was involved in all

cases [33] and that *Zea* sperm had successfully reached the embryo sac at the time of normal fertilisation. The non-random transfer of *Zea* genes or the non-random incorporation of selected *Zea* DNA fragments into specific positions of the *Tripsacum* genome were suggested. For such events to occur, reproductive abnormalities in the egg parent would seem to be essential. Seed parents of F1 Alborea show much-reduced pollen and produce 2n pollen [21]. The genetic control of the absence of cytokinesis after restitutional meiosis in 2x alfalfa eggs was studied by Baraccia et al. [42] and five genes were proposed to control the production of an unreduced gamete among members of the *M. sativa* subspp. complex. Chromosome doubling by unreduced gametes has undoubtedly played an important role in the evolution of autoploidy in alfalfa, but its role in facilitating hybrid embryo development between different species in the genus *Medicago* has been undetermined until the work with Alborea [23,34].

Another cultivated autotetraploid with a large number of related wild and cultivated species at different ploidy levels is the potato, *Solanum tuberosum* (2n = 4x = 48). Sexual polyploidisation through unreduced gametes has been a significant factor in its evolution [43]. Boyes and Thompson [44] and Valentine [45] established that as well as 2n gametes, the need for a balance of chromosome sets between maternal tissue, the embryo, and endosperm was required for successful interploidy crosses. It would appear that the endosperm of M8 and MB Alborea hybrids are able to tolerate an unbalanced genome dosage. The Endosperm Balance Number (EBN) hypothesis, developed in the early 1980s and reviewed by Carputo et al. [46], postulates that each species has a genome-specific ploidy, the EBN, which must be in a 2:1 maternal-to-paternal ratio in the hybrid endosperm for normal endosperm development. With unreduced 4x eggs in M8 and MB and normal reduced 2x *M. arborea* pollen, it could be anticipated that the Alborea endosperm could be 10x (4:1 EBN ratio) compared with 6x (2:1 EBN ratio) from a normal 4x × 4x cross. These EBN values are only theoretical; the actual EBN of the Alborea hybrids remains undetermined. However, the postulated higher maternal-to-paternal genome ratio may have facilitated endosperm development, which is a necessary precursor to embryo development.

7. Use of Alborea in Alfalfa Breeding

Nine distinct germplasm sources have been recognised and analysed within the *M. sativa* subspp. complex [47]. Since 1971, the alfalfa cultivars released in North America typically include genetic variations from all nine germplasm sources [47]. Kidwell et al. [48] conducted an RFLP evaluation of these nine germplasm sources and only two, *M. sativa* subsp. falcata cv WISFAL and Peruvian, formed distinct clusters; the remaining seven sources were not clearly discriminated by the analysis. Recent molecular marker studies showed that 4x alfalfa populations maintained high levels of within-cultivar genetic diversity [49]; however, alfalfa yields have stagnated in North America and Australia [6–8]. This has provided the impetus to attempt to introduce new traits from other *Medicago* species into the *M. sativa* subspp. complex, which will enhance alfalfa productivity and persistence. *M. arborea* is one such species that has many traits that would increase the versatility of alfalfa (Table 1). It can grow to 4 m in height, has a shrubby growth habit, is drought- and salt-tolerant, and is the longest-lived of all the *Medicago* species. In addition, its seeds are four times larger than alfalfa (100 per gram versus 400 per gram) [3].

In a test of transmission of a single *M. arborea* trait from a yellow-flowered Alborea plant, yellow pigment was backcrossed into the alfalfa cultivar Pegesis (Bingham, unpublished data). Pegesis alfalfa has a purple flower colour; the addition of the yellow pigment resulted in variegated flowers due to the co-expression of purple and yellow. The variegated plants that were selected backcrossed three generations and did not express other *M. arborea* traits, indicating the gene for yellow pigment was likely carried on a short segment of *M. arborea* chromatin. The pollen and seed production of variegated plants was normal. The behaviour of yellow in the backcross was consistent with yellow incorporated in an alfalfa chromosome.

Some *M. arborea* accessions were shown to be resistant to races 1 and 2 of *Colletotrichum trifolii* [50] and to *Phytophthora medicaginis* (Irwin, unpublished data). Armour et al. [27] reported a transfer of resistance to *C. trifolii* race 2 from *M. arborea* to *M. sativa*. The resistance in the Alborea hybrid was successfully transferred to testcross progeny (Alborea x susceptible *M. sativa* clone), with 35% of the testcross progeny resistant. The resistant testcross plants were polycrossed and the resistance was again transferred to the polycross progeny. This indicated that the anthracnose resistance from *M. arborea* was stable and transmissible through at least two generations. It has subsequently been utilised in developing experimental alfalfa synthetics.

Alborea and Alborea × alfalfa crosses have been agronomically evaluated in a range of experiments conducted across locations in different continents. Irwin et al. [32] reported yield increases of up to 42% over adapted cultivars in the subtropics at Gatton, Queensland. Humphries et al. [25] tested selections of Alborea 101 in a Mediterranean environment in South Australia. One selection, CTA 033, had an estimated 23–30% higher yield than the commercial control cultivars. In field evaluations in Mediterranean environments in South Australia and Chile, an Alborea hybrid derived from the cross *M. sativa* cv Genesis x Alborea showed one of the highest dry matter yields and persistence levels [26]. Based on the above, evidence is emerging that Alborea has the capacity to enhance alfalfa productivity and persistence, at least in subtropical and Mediterranean environments.

Tani et al. [51] researched responses to salt shock and stress in seedlings of alfalfa, *M. arborea*, and their hybrid (Alborea). The two parental species appeared to regulate different components of the salt-tolerance mechanism. The Alborea population studied was more sensitive to all salt treatments than its parents, except the low dose (50 mM NaCl). This was most likely due to no selection for salt tolerance being undertaken during the development of the Alborea population. However, it should be possible to pyramid salt-tolerance genes in individual plants by cycles of selection for salt tolerance.

8. The Future

While each Alborea plant contains less than half of the *M. arborea* genome, it has been shown that introgressions have occurred from at least three of the *M. arborea* chromosomes [32,52]. Thus, so long as Alborea populations generated from several different hybrid individuals are used in breeding, it should be possible to transfer most of the *M. arborea* traits to alfalfa. The use of DNA marker technology, as described in Reference [53] and specific to the *M. arborea* genome, would facilitate the identification of the partial hybrids. Currently, only two alfalfa seed parents (MB and M8) and their progeny have been successfully used to generate hybrids [21,23]. The genome of MB codes for winter dormancy and low yields; its derivatives were relatively low yielding in Australia [32]. However, it should be possible to breed higher-yielding alfalfa seed parents by crossing M8 or MB, for example, with adapted genotypes and selecting for the reproductive abnormalities of both unreduced eggs and pollen. Unreduced pollen, due to its much-increased size, can be readily identified, and it provides an effective screening methodology to identify seed parents that also produce unreduced eggs, which appears to be essential for hybrid development. MB, M8, and all Alboreas examined produce unreduced eggs and pollen, and it is easier to screen for unreduced pollen than for unreduced eggs [23].

Having being derived from alfalfa parents with reproductive abnormalities, Alborea plants vary in the amount of pollen they produce, from a trace to about 25% of normal alfalfa [23,27]. Female fertility is expressed at a high level when pollinated with 4x normal *M. sativa*. This indicates that Alborea lines, when used as seed parents, could be useful in semi-hybrid development, as proposed by [54,55]. In Wisconsin and Queensland, work continues to identify adapted alfalfa cultivars/lines that complement Alborea selections for yield and persistence. Recent work has demonstrated the capacity for Alborea crosses with alfalfa to enhance alfalfa yield and persistence in Mediterranean environments, which are characterised by drought in summer [25,26]. Kang et al. [56] identified traits in alfalfa that provided tolerance to drought. These traits included lower-leaf wilting, delayed

senescence, and leaf tolerance to desiccation under stress, smaller leaves, lower early root and shoot growth, and lower stomata density. Screening Alborea for the above traits, which exist in *M. arborea*, and their use in breeding may facilitate the development of more drought-tolerant alfalfas. Del Pozo [57] recently reported two Alborea selections to be the most drought-tolerant and productive entries in an alfalfa diversity panel tested in a Mediterranean environment in central Chile.

It has been shown that specific *M. arborea* alleles and traits can be transferred from the hybrids to alfalfa [21,25–27,32], indicating a degree of stability for the alleles/traits studied. However, as no genomic in situ hybridisation (GISH) studies have been performed on partial hybrids, it is not known whether chromosome fragments from *M. arborea* become integrated into the alfalfa genome or if a chromosome from *M. arborea* is transferred. It could be expected that a univalent would not be meiotically stable. Stability has been demonstrated for several traits, including anthracnose resistance, winter activity, and flower colour, and work is continuing for the other traits listed in Table 1. The results suggest that fragments of the *M. arborea* chromosomes have introgressed into the chromosomes of the *M. sativa* seed parent, providing meiotic stability. The high proportion (7%) of AFLP bands that were unique to the hybrid [27] was indicative of genome rearrangement, as could be expected with the integration of fragments of *M. arborea* chromosomes, which are more likely to be meiotically stable than univalents. Future research should utilise GISH studies to increase the understanding of the cytogenetic basis of partial hybridity. Pyramiding *M. arborea* traits from Alborea into adapted alfalfa cultivars may provide opportunities to increase the productivity, persistence, and utilisation of this important forage legume.

9. Conclusions

The importance of reproductive abnormalities, including unreduced eggs, in the *M. sativa* seed parents to the generation of partial hybrids between *M. sativa* and *M. arborea*, with a predominance of the *M. sativa* genome, was experimentally established. Although each hybrid contained about 5% of the *M. arborea*-specific alleles, it was possible to transfer a range of *M. arborea*-specific traits to the hybrids, which might have value in alfalfa improvement. Such traits included a larger seed size, indeterminate growth, frost tolerance, and heterosis for productivity and persistence in crosses with alfalfa. It is possible to conclude that the reviewed research conducted over the last 20 years has demonstrated the potential of the interspecific partial hybrids to enhance alfalfa improvement through long-term breeding activities.

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