

Review

# Factors Underlying Seed Yield in Red Clover: Review of Current Knowledge and Perspectives

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**Abstract:** Red clover is a valuable forage crop, but often copes with unsatisfactory seed yield. Management practices to increase seed yield include fertilization, adequate weed and pest control, the synchronization of flowering through pre-cutting in spring, and the application of plant growth regulators to prevent lodging. Seed yield problems may have variable underlying reasons, such as inadequate pollination, fertility or genetic problems, or a combination of such traits. In this review paper, we summarize the results of recent publications that shed new light on the traits explaining seed yield differences between red clover genotypes. The main seed yield components are the number of flower heads per plant and the seed yield per flower head. Seed yield differences between diploid and tetraploid red clover are largely explained by the lower seed number per head. Recent research showed that, although inadequate pollination can lead to poor seed yield in certain areas, pollination cannot explain differences in seed yield between genotypes, cultivars, or ploidy levels. Correspondingly, corolla tube dimensions are not associated with seed yield, in spite of what is often believed by seed producers. On the other hand, fertility problems such as aberrations during male meiosis tend to occur more frequently in tetraploid genotypes and/or genotypes with low seed yield. A recent genetic study revealed 34 candidate genes for seed development, which opens perspectives for marker-assisted breeding. A final and remarkable finding is the occurrence of self-fertility in tetraploid red clover and its association with high seed yield. Breeders should be aware that selection for seed yield in tetraploid red clover may lead to unintentional selection for self-fertility, with possible consequences for inbreeding. The implications of recent findings for seed yield breeding and for the creation of novel tetraploids are discussed. Future research opportunities are considered.

**Keywords:** *Trifolium pratense*; pollination; flowering traits; fertility; seed development; breeding

## 1. Introduction

Red clover (*Trifolium pratense* L.) is a perennial forage legume belonging to the Fabaceae family. This crop is the second largest forage legume crop in the world in terms of production area, after alfalfa (*Medicago sativa* L.) [1]. It is grown in temperate areas of the world, commonly in combination with grasses such as timothy (*Phleum pratense* L.), meadow fescue (*Festuca pratensis* Huds.), tall fescue (*Festuca arundinacea* Schreb.), and/or perennial ryegrass (*Lolium perenne* L.) [2]. Red clover has the ability to fix atmospheric nitrogen through symbiosis with the bacteria *Rhizobium leguminosarum* biovar *trifolii*, which allows it to attain high forage yields (up to 15 tons DM/ha in monoculture)—higher than most forage grasses—even without N fertilization. As a consequence, red clover is a

valuable rotational crop, especially in organic agriculture where no synthetic N fertilizers are used [3]. Red clover has a crude protein content of around 18% (similar to alfalfa and white clover), which remains stable throughout the growing season, in contrast to forage grasses [2]. Moreover, red clover increases palatability and digestibility of the forage mixture, resulting in higher weight gains and reproduction rates of cattle [4,5]. When red clover is included in the ruminant's diet, it provides higher crude protein and polyunsaturated fatty acids content in the final product (meat and milk) [4].

Red clover is, by nature, a diploid species ( $2n = 2x = 14$ ), but tetraploid ( $2n = 4x = 28$ ) varieties also exist in commercial production. Tetraploid red clover was first produced in 1939 by treating germinating seeds with colchicine [6,7]. Other chemicals that can be used to generate tetraploids through chromosome-doubling are trifluralin, oryzalin, and nitrous oxide gas. Alternatively, tetraploids can be obtained by gametophytic non-reduction, i.e., by crossing one diploid plant and one tetraploid plant [8]. The first generations of newly created tetraploids usually suffer from inbreeding, through the partial loss of heterozygosity during the chromosome-doubling event. Intercrossing of tetraploid genotypes in subsequent generations restores heterozygosity. Tetraploid red clover generally attains up to 20% higher forage yields and is generally more tolerant to biotic and abiotic stresses compared to diploid red clover [6,9,10]. The downside of tetraploid cultivars is the often considerably lower seed yield [9,11,12].

The commercial success of any cultivar depends heavily on a reliable supply of competitively priced seed [11]. Yields of 500 kg seed/ha are considered satisfactory in large-scale productions, but such yields are often not attained [13]. In Western and Central Europe, diploid red clover cultivars typically produce around 400–500 kg seed/ha, depending on the harvest year, location, and cultivar [6]. In Oregon (USA), seed yields are usually higher: Between 600 to 1200 kg/ha [14].

Tetraploid cultivars produce in general 200–400 kg seed/ha [6,9], which is 20–50% less seed compared to diploids [2,9,15]. Numerous cultivars, especially tetraploids, but also diploids, are not commercialized in spite of their agronomic superiority [11]. In addition, various publications report that seed yield in red clover has decreased in the past decades [9,16–20]. In Sweden, seed yields of red clover have declined and became more variable since the 1980s [20]. In order to keep red clover on the market against competitive prices, seed yield needs to be improved, especially in tetraploid cultivars.

Given its relevance for breeders and farmers, in recent years, several studies have investigated diverse aspects of seed yield in red clover. It remains unclear, however, if unsatisfactory seed yields are due to poor cultural practices during seed production, inadequate pollination, fertility problems, or any other genetic problems. In this paper, we summarize the results of recent publications that shed new light on the complex process of seed yield in red clover and discuss possible directions of future research and venues to improve seed yield in red clover through breeding.

## 2. Reproductive Characteristics of Red Clover and Implications for Breeding

Red clover is an obligate out-crossing species with a strong gametophytic self-incompatibility controlled by a single S-locus and a severe inbreeding depression [2,6,21]. The diversity of S-alleles in red clover is huge: Populations are estimated to harbor between 143 and 193 unique S-alleles [22]. Due to the out-crossing character of the species, red clover populations display a high phenotypic and genotypic diversity, and intra-population variation is often larger than inter-population variation [6]. Breeding methods in red clover rely on open pollinated populations: Mass selection, family selection (or recurrent phenotypic selection), or polycross selection. These methods have helped to improve agronomic traits such as forage yield, winter hardiness, and resistance to certain diseases, but have been less successful for the improvement of seed yield [6,23].

The inflorescences of red clover are formed at the tips of the stems and consist of spherical heads composed of 50–200 individual flowers [24–26]. The length of the corolla tube is approximately 10 mm, with nectaries located at its base [25]. Flowers are generally pink, although pigmentation can range from white to purple [26]. The pistil and 10 stamens extend through the entire corolla tube, with the stigma and anthers enclosed by the keel petals [25]. Each ovary contains two ovules, but usually only one develops into a mature seed [25]. Red clover is insect-pollinated: A pollinator forcing

its head into the corolla exerts pressure on the keel petals, which causes the stigma and anthers to “trip” and touch the insect [25]. Wild bumblebees (*Bombus* spp.) and honeybees (*Apis mellifera* L.) are the most important pollinators of red clover, but different species and subspecies are important in different regions [25].

Bumblebees with long tongues such as *B. pascuorum* ssp., *B. ruderatus*, and *B. hortorum* ssp. are considered the most efficient pollinators of red clover, as their tongues can easily reach the nectar at the bottom of the corolla tube [19,24]. The effectiveness of long-tongued bumblebees became evident when red clover seed yields increased drastically after their introduction in New Zealand around 1885 [27]. Short-tongued bumblebees such as *B. terrestris* L. and *B. lucorum* L. are suboptimal, because they often fail to reach the nectar through the corolla tube [16,19,24,28,29]. Instead, short-tongued bumblebees can bite holes in the lower part of the corolla to “rob” the nectar without carrying out pollination, referred to as “nectar robbing” [24,25,28,29]. In this regard, Hawkins [30] found that seed yields of red clover were closely correlated to the abundance of long-tongued bumblebees, while the abundance of short-tongued bumblebees was not related to seed yield. Keeping in mind that bumblebee nests usually house fewer than 100 individuals, recommendations were made to grow red clover seed crops in small fields and in regions rich in bumblebee populations [31].

The ability of honeybees to pollinate red clover has long been questioned, as their short tongues render it difficult to reach the nectar [24]. Nonetheless, in hot and dry weather conditions, nectar rises sufficiently high in the corolla tube for honeybees to access it [32]. Also, when few alternative pollen and nectar sources are available, honeybees are observed as pollinators on red clover [12,24,33,34]. To conclude, honeybees are the main pollinators of red clover in warm climates such as Serbia [13], while bumblebees prevail in temperate climates such as Belgium [35] and in Nordic regions such as Denmark and Sweden [12,36]. Leafcutter bees (*Megachile rotundata* Fab.) are common pollinators of red clover in the western part of North America [37].

### 3. Steps Undertaken to Increase Seed Yield in Red Clover

#### 3.1. Improvement of Agricultural Practices

In temperate and warm regions, red clover seed production fields are usually established in late summer or autumn, and harvested for seed in the two following years [11]. In most Nordic regions, red clover seed crops are seeded in spring, together with spring wheat, barley, or oat as a cover crop, and harvested for seed in the two following years [11].

Management practices that can impact seed yield are, for example, fertilization with phosphorus and potassium (depending on the soil nutrient level and exploitation of the crop) [2], fertilization with boron in B-deficient soils [38], irrigation in arid areas [39,40], and adequate weed and pest control [19]. Clover seed weevils (*Apion trifolii* and *Apion apricans*) and the lesser clover leaf weevil (*Hypera nigrirostris*) can cause heavy seed losses in some years and some locations [41]. Monitoring such major insect pests from the budding stage to the end of flowering allows timely insecticide application [42].

The most commonly applied practice in temperate and warm regions is pre-cutting in spring at the onset of flowering to synchronize flowering [2,43]. In addition, this lets flowering coincide with the peak in pollinator activity [44] and reduces vegetative biomass in the next cut, making seed harvest and threshing easier. In cold climates, such as most Nordic regions, pre-cutting in spring is not recommended due to the already short growing season, as it would excessively delay flowering and seed ripening [11].

A recently optimized practice is to apply plant growth regulators (PGRs) such as Trinexapac-ethyl—commercially registered as Palisade® EC or Moddus®—which inhibit gibberellic acid biosynthesis, resulting in shorter plants, firmer and more upraised stems, more intensive flowering (26–62% more flowers per square m) and easier harvesting [39]. When applied at stem elongation (BBCH 32), increases in seed yield of 9–21% have been reported [39,45,46]. Unfortunately, the effectiveness of Trinexapac-ethyl heavily depends on the accurate timing of the application, the weather conditions after application, and the crops’ stress level [45].

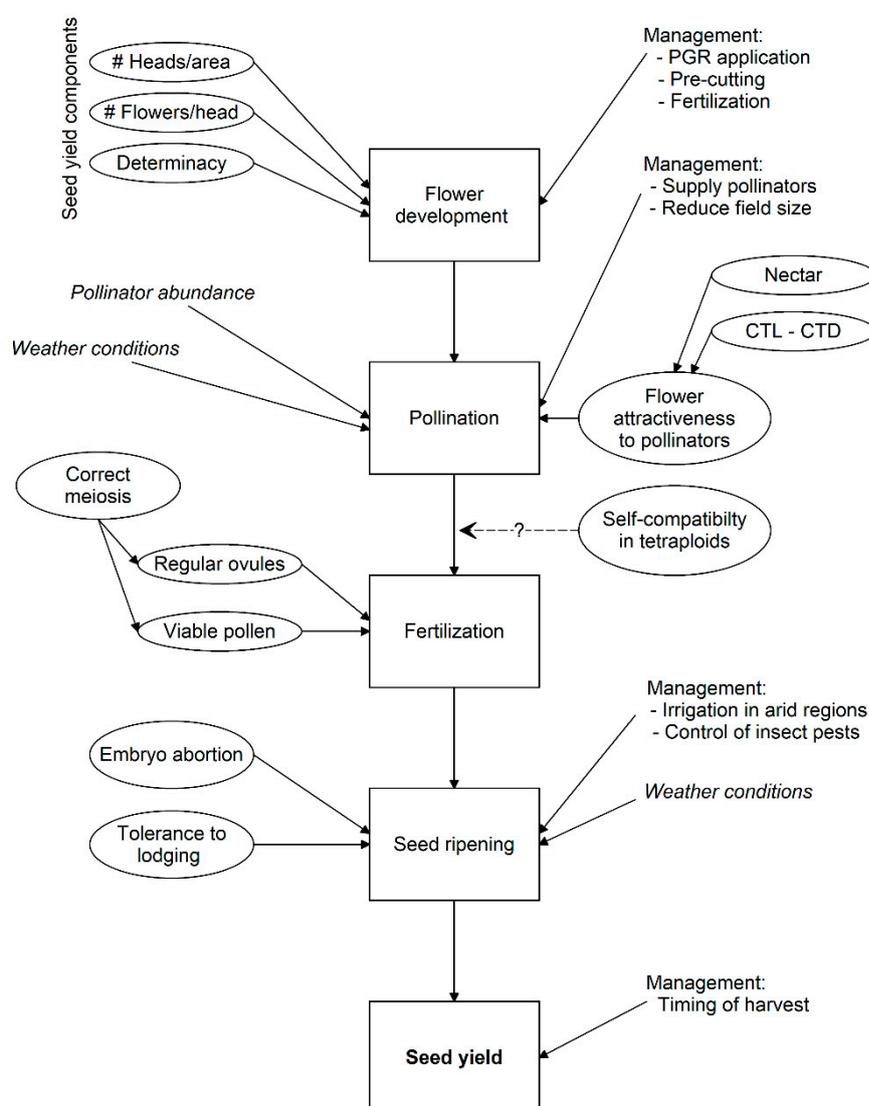
Another common practice is to increase the number of pollinators by supplementing them [47]. The supplementation of pollinators is justified in warm climates, in large seed production fields, or in regions where bumblebee populations have declined [19,20,32,48]. Seed producers often prefer to supplement honeybees instead of bumblebees: Bumblebee nests are commercially available, but it is uneconomical to provide enough bumblebees to ensure adequate pollination. Moreover, in Europe, only *Bombus terrestris* L. is commercially available, yet this bumblebee is a poor pollinator of red clover as it often robs nectar [24,25]. Red clover seed producers typically supply two to three honeybee hives per hectare during flowering, sometimes more [49,50]. Seed yield increases up to 34 kg/ha are reported, although studies that statistically investigate the effect of supplementing honeybees on seed yield are not available [50]. The effect of supplementing honeybees is most likely region-dependent. Jevtic [51] reported that treating red clover with sugar syrup at full flowering attracted more honeybees, leading to 20% higher seed yields. Nonetheless, the effects of supplementing or attracting honeybees is often questioned, as honeybees can act as competitors for long-tongued bumblebees: Reduced numbers of long-tongued bumblebees have been reported on red clover when honeybee hives are supplemented [19,47].

### 3.2. Breeding for Higher Seed Yield

Seed yield is, next to forage yield, disease resistance and persistence, a major breeding goal in red clover [6,23]. A typical breeding program of red clover relies on open pollinated populations, with selection of individual genotypes for vegetative traits in the first year(s) and selection for seed yield in the final year of the selection trial [6]. In spite of the breeders' efforts, seed yield in red clover has not improve substantially in the last decades [11,52]. Several factors can explain this. First, seed yield assessed on spaced plants is a poor predictor of seed production capacity in field conditions in forage crops [23]. The assessment of a genotype's breeding value for complex traits such as seed yield is difficult, as only one plant per genotype is evaluated. Instead, it would be more efficient to select for seed yield in plots of progenies from elite genotypes, or to assess the seed yield potential of breeding material early on by establishing seed production trials. Both options are rarely done because they would require substantially more time and effort. Second, strong selection for forage yield and persistence leaves perhaps little room for improvement of seed yield, since both traits are negatively correlated with seed yield [53,54]. Third, progress for seed yield (as for other traits) might be slowed down because in a typical breeding trial, the highest seed-yielding genotypes selected for the next breeding cycle have been pollinated to a large extent by non-selected genotypes with unsatisfactory seed yield. While in the first two cases, adaptation of the breeding scheme and selection criteria are the only option, it has been shown that marker-assisted parentage analysis enables to discriminate progeny plants that result from a cross between two high seed-yielding genotypes, offering opportunities to select the best progeny to advance to the next generation. For example, in diploid red clover, parentage analysis resulted in more efficient breeding for seed yield when applied in a family selection trial: Seed yields were 23% and 76% higher compared to traditional family selection in two successive harvest years [55]. Also, in tetraploid red clover, marker-assisted parentage analysis was able to identify progeny plants that were the result of pollination by a high seed-yielding genotype, but this did not help to improve seed yield further [56]. One of the main differences between both studies above was that in the former case, a first cycle selection trial was assayed, while in the latter case, a second cycle selection trial was tested, meaning that the tetraploid genotypes had undergone a first round of selection which might have reduced the variation available. The heritability of seed yield in this tetraploid population was very low [56], indicating that mainly non-additive genetic effects were responsible for the differences observed. Further exploration of this methodology in other tetraploid selection trials is required to formulate more definitive conclusions.

#### 4. Factors Explaining Seed Yield in Red Clover

Figure 1 illustrates the process of seed formation in red clover and the various factors that affect it, including plant traits (under genetic control), management practices, and environmental factors.



**Figure 1.** Process of seed formation in red clover with the underlying plant traits (circular boxes), management factors that can be applied to enhance seed yield, and environmental conditions (italic) that influence it. PGR = plant growth regulator; CTL = corolla tube length; CTD = corolla tube diameter.

##### 4.1. Seed Yield Components

Multiple studies have suggested that the number of flower heads—or head number (HN)—per plant or per unit area is the critical factor for seed yield in red clover [40,54,57]. Likewise, the QTL (quantitative trait loci) study of Herrmann [58] revealed that in diploid red clover, HN was the major seed yield component explaining seed yield per plant ( $r = 0.89$ ), along with the seed number per head (SN/H) ( $r = 0.47$ ). Dijkstra [59] showed that diploid plants had, on average, 7.4% two-seeded pods vs. 2.5% in tetraploids. There was a moderate correlation between seed yield per plant and the fraction of two-seeded pods in diploids ( $r = 0.35$ ), but a poor correlation in tetraploids ( $r = 0.08$ ).

A detailed analysis of seed yield components at the genotype level in diploid and tetraploid red clover was carried out recently [26,60,61]. The three studies pinpointed HN and SN/H as the most important components of seed yield in both diploids and tetraploids, with HN and SN/H explaining

together up to 98% of the variation for seed yield per plant in tetraploids [60]. However, regression modeling revealed that seed yield per plant depends most on HN in diploids (62%), but on SN/H in tetraploids (62%) [26]. On average, tetraploid genotypes produced fewer seeds than diploids, which was largely explained by the lower SN/H (43.4 vs. 8.9 seeds/flower and 8930 vs. 1487 seeds/plant in diploids and tetraploids, respectively) [26,60]. In addition, tetraploid genotypes displayed fewer ripe flower heads at seed harvest than diploids (159.2 vs. 209.7 heads, or 73.1% vs. 78.3%, respectively), which indicates a slightly lower degree of branching and determinacy in tetraploids [26]. Amdahl [9] found similar results when studying seed yield components in dense stands of 12 tetraploid cultivars and populations from Scandinavia. A high correlation between seed yield  $m^{-2}$  and the number of flower heads  $m^{-2}$  was observed in two experiments ( $r = 0.956$  and  $r = 0.977$ ).

To conclude, recent studies have identified HN and SN/H as major determinants of seed yield in single plants and in dense stands of red clover [9,26,58,60,61]. Lower seed yield in tetraploids than in diploid genotypes is mostly explained by the lower seed number per head, and only partly by the lower number of ripe flower heads at seed harvest. The substantially lower seed number per head in tetraploids may indicate either inadequate pollination or problems with fertility. Both possibilities are discussed in the following sections.

#### 4.2. Inadequate Pollination

Poor seed yields in red clover are often ascribed to inadequate pollination. It has been hypothesized that the declining abundance of bumblebees and wild pollinators in nature is partly responsible for the reduced seed yields observed recently [17]. In Denmark [48] and Sweden [20], for example, the abundance and species-richness of long-tongued bumblebees on red clover has severely declined since the 1940s, while short-tongued bumblebees have become more dominant. The change in pollinator communities in Sweden may explain the decline in seed yield and yield stability of red clover observed in recent years [20]. Pollination problems may be aggravated by a changed practice towards larger seed production fields, where pollination may become insufficient in the central part of the field due to reduced accessibility for the insects [31].

Other studies argue that the length of the corolla tubes can be the problem, especially in tetraploid red clover, given that tetraploid flowers are usually longer than diploid flowers. The nectar at the bottom of long corolla tubes would be less accessible for pollinators, resulting in inadequate pollination and low seed yield [25,62,63]. The “corolla hypothesis” likely originated in New Zealand where, before the introduction of European bumblebees around 1885, red clover often failed to set seed in the presence of the short-tongued native bees and honeybees [27,64,65]. The observation of nectar robbing spurred the corolla hypothesis [24,30,31]. Short-tongued bumblebees such as *Bombus terrestris* L. act as primary robbers and puncture the corolla tube to access the nectar [24]. Afterwards, secondary robbers such as honeybees, unable to bite through corolla tubes themselves, rob newly formed nectar through the bite holes as well [24]. Consequently, numerous flowers are not pollinated and deprived of nectar, thus rendered less attractive for long-tongued bumblebees [16,66].

There is some evidence for the corolla hypothesis. For example, Hawkins [67] observed that among eight red clover cultivars, cultivars with the shortest corolla tube length (CTL) were more frequently visited by bumblebees and produced more seeds than cultivars with the longest CTL. Bumblebee species with short tongues preferred to pollinate cultivars with shorter CTL, whereas corolla biting most frequently occurred in cultivars with longer CTL, presumably because less of their nectar had been removed [67]. However, most studies that support the corolla hypothesis only argue that the CTL in tetraploids is larger than in diploids [68,69]. For example, Bingefors [70] reported that the tetraploid cv. “Ulva” produced fewer seeds than its diploid ancestor cv. “Ultuna”, which they attributed to the 25–30% lower flower head production per unit area and to the 0.74 mm longer CTL in “Ulva”. Congruently, Bender [44] found that two tetraploid cultivars displayed 10% longer (and 30% wider) corolla tubes compared to their diploid ancestor cultivars (the ones that had been submitted to tetraploidization). In this case, it was demonstrated that higher proportions of long-tongued bumblebees (51.9% vs. 37.3%), lower proportions of short-tongued bumblebees (37.6% vs.

40.1%), and more frequent nectar robbing (11.1% vs. 4.6%) occurred on the tetraploid cultivars compared to their diploid ancestors [44].

There also exists a plethora of studies whose results do not support the corolla hypothesis. For example, Starling [71] observed no association between CTL and the seed number per head among 218 diploid genotypes derived from crosses between cultivars with diverging CTL: The cultivar with the shortest CTL actually displayed the lowest seed yield. Hawkins [72] stated that nectar robbing was not likely to be associated with the low seed setting of red clover, while Julén [73] stated that the tendency of bumblebees to rob nectar varies greatly between years and along the flowering period. Often, nectar robbing intensifies in late summer and autumn (personal observation), possibly because of few alternative nectar sources for pollinators in that period. Brodsgaard [16] observed that the CTL depended more on the location and management of the seed crop than on the ploidy level. The diploid cultivar “Rajah” had average CTLs of 8.71 mm in a conventional field vs. 8.82 mm in an organic field, while the CTL was 8.75 mm in the tetraploid cultivar “Kvarta” (conventional field). The degrees of corolla biting/nectar robbing were 3.6%, 13.4%, and 17.2% in the three fields, respectively [16].

In the above-mentioned studies, which suggest inadequate pollination as reason for low seed yield in tetraploid red clover, measurements were performed at the population level [44,68–70]. Yet, given the large genetic diversity within red clover cultivars, comparing average cultivar values often does not reveal hidden correlations between seed yield, flowering traits, and pollination preference, and may lead to faulty conclusions. Recent studies at the genotype level have provided different insights. Three recent studies found that tetraploid genotypes indeed have longer CTL than diploids [26,60,61], but a very large overlap in CTL between ploidy levels was also found. In addition, the corolla tube diameter (CTD) is larger in tetraploids than in diploids [26]. Importantly, these three studies found no association between CTL and seed yield. In fact, some genotypes with the largest CTL actually displayed the highest seed yields [60]. Hence, recent studies at the genotype level strongly contradict the corolla hypothesis [26,60,61]. Longer corolla tubes appear to be also wider, as observed by [26,74], which may allow pollinators to insert their heads deeper into the flower and reach the nectar in spite of the increased CTL. It is therefore not surprising that breeding for reduced CTL did not lead to great improvement in seed yield in the work of Hawkins [72]. Jablonski [75] reported increased seed yield in a population derived from a mutant genotype with short corolla tube. The population had a 24% shorter corolla than the cultivar “Nike”, but also produced 29% more flower heads  $m^{-2}$  [75]. It is therefore impossible to determine which factor (CTL or number of flower heads  $m^{-2}$ ) contributed most to the increase in seed yield in this work.

In addition, no correlation has been found either between seed yield and other flowering traits such as the flower number per head (112 in diploids vs. 115 in tetraploids) or flower pigmentation [26]. Furthermore, Vanommeslaeghe [35] investigated whether pollinator preferences explained differences in seed yield between diploid and tetraploid red clover at the genotype level. Nectar production, nectar concentrations of glucose, fructose, sucrose, and total sugar in the nectar differed among genotypes with known differences in seed yield, but did not differ between ploidy levels, and were not associated with seed yield. In pollinator preference binary-choice experiments with *B. pascuorum* L., no bumblebee preference was recorded either for ploidy or seed yield, with the exception of a single tetraploid genotype that stood out for its high nectar production (10.3  $\mu$ L per 10 flowers compared to 3.5  $\mu$ L in the average tetraploid). The latter genotype was more attractive to *B. pascuorum* L., but actually displayed low seed yield, suggesting that factors other than pollination are responsible for its low seed yield [35].

Recent findings at the genotype level were confirmed by recent studies at field scale. A study in a seed production field of the tetraploid cv. “Avanti” confirmed the lack of correlation between CTL and seed yield when the analysis was done at the genotype level [74]. Jing [12] found similar visitation frequencies of honeybees on the diploid cv. “Rajah” and the tetraploid “Amos”. However, the SN/H was 68.7% higher in “Rajah” than in “Amos”, indicating a lower seed setting ability in the tetraploid cultivar under the same pollination conditions. Vanommeslaeghe [35] confirmed in a field

experiment that *B. pascuorum* L. does not discriminate between diploid vs. tetraploid genotypes, nor between genotypes with high vs. low seed yield.

In conclusion, pollination in red clover under field conditions is complex, as it probably depends on the prevailing bumblebee/honeybee (sub)species, competing pollen and nectar sources, and local management strategies, which all differ from region-to-region and from year-to-year. For honeybees alone, there exist at least 29 subspecies [76]. Tongue length can vary substantially between subspecies, but even between colonies of the same subspecies, as observed by Brodsgaard [16] in “Buckfast” honeybees. Unfortunately, the prevailing pollinator subspecies and its tongue length is rarely reported in publications. While the corolla hypothesis may be valid in some regions or circumstances, it must not be generalized. Pollination can probably explain poor seed yield in some locations, e.g., where insufficient pollinators are present. However, neither pollinator preferences nor corolla tube dimensions can explain differences between genotypes, cultivars, or ploidy levels.

### 4.3. Fertility Problems

Reduced fertility may lead to seed yield problems in red clover. Especially in tetraploid red clover, fertility problems often remain high in spite of ample breeding efforts, as is often observed in induced autotetraploids [77]. Reduced fertility may have various underlying reasons, including (1) deficient meiosis which may lead to (2) poor pollen quality, (3) embryo abortion during seed development, and possibly (4) altered self-incompatibility interactions in tetraploid red clover. In red clover, only a limited number of studies is available on fertility traits, and only few of them have investigated fertility traits in relation to seed yield at the genotype level.

Aberrations in meiotic division could lead to unviable pollen and ovules, with reduced fertility as a consequence [78]. Meiotic aberrations were observed in tetraploids by Büyükkartal in tetraploid red clover [79], but no link was made to seed yield. Vleugels [80] analyzed male meiosis among 19 diploid and 17 tetraploid genotypes with well-characterized and diverging seed yields: Tetraploid genotypes revealed more meiotic aberrations than diploids, and there was a trend of decreasing seed yield at increasing frequencies of meiotic aberrations. Analyzing a larger group of genotypes may allow to statistically prove an association between seed yield and meiotic aberrations. In certain low seed-yielding genotypes, high frequencies of meiotic aberrations (up to 56% aberrations) were observed, which suggests that in these genotypes, defective (male) meiosis may be a major cause of low seed yield [80].

Pollen quality was studied by Büyükkartal [81] in tetraploid red clover and compared between diploid and tetraploid cultivars [77] and genotypes [80] in later studies. Büyükkartal [81] found 57% pollen germination in a Turkish wild tetraploid red clover population. Grebrenisan [77] found that tetraploid cultivars produced pollen with lower viability and lower germination rates than diploid cultivars. Vleugels [80] observed a similar trend for pollen germination, although it is difficult to compare absolute values due to different culture media used in these studies. Vleugels [80] also noted that pollen viability was strongly influenced by the weather conditions during the three days before pollen harvest.

Embryo abortion is a commonly observed phenomenon in legumes, and has been the focus of various studies in forage legumes [80,82,83]. In diploid red clover, Povilaitis [84] found that 70% of ovules developed into mature embryos in high seed-yielding genotypes vs. 40% in low seed-yielding genotypes. In addition, flowers of high seed-yielding genotypes often raised two embryos, while flowers of low seed-yielding genotypes often contained no embryo at all. This indicates that low seed-yielding genotypes can simply support fewer developing seeds. In a more recent study in a tetraploid red clover population from Turkey, only 18% of the flowers raised one or two embryos, and merely 5.8% of the ovules developed into mature seeds [85]. Vleugels [80] studied embryo development in 11 genotypes (4 diploids, 7 tetraploids) with well-characterized, divergent seed yields. They noted high degrees of embryo abortion in most genotypes, but did not find a correlation between embryo abortion and seed yield. A breakdown of self-incompatibility is commonly observed in polyploid species [86], and has been described in tetraploid red clover as well [77,87–91]. Povilaitis [84] suggested that low seed-yielding red clover genotypes contain genetic factors that suppress pollen

tube growth for both own and foreign pollen. Grebrenisan [77] studied pollen tube growth in detached red clover pistils. When the pistils were self-fertilized, a higher fraction of growing pollen tubes was observed in tetraploids than in diploids (12% vs. 3%, respectively). In both ploidy levels, however, self-fertilized pistils became more self-fertile when they were fertilized again with own pollen [77]. In other words, repeated self-pollination in the absence of other red clover pollen may inactivate self-incompatibility barriers. Vleugels [80] found that high seed-yielding genotypes self-fertilized to a large extent (83% on average), whereas no self-fertilization at all was detected in low seed-yielding tetraploids or in diploids. A subsequent study confirmed that self-fertilization is common in tetraploid red clover that has been pre-selected for seed yield and synchronous flowering [56]: 3 out of the 14 highest seed-yielding genotypes were self-fertile with individual selfing rates up to 20%. These observations suggest that self-fertility in tetraploid red clover acts similarly as in the autotetraploid alfalfa, where selfing rates average 11.8% in open-pollinated fields with individual selfing rates up to 52.2% [92].

As discussed by Vleugels [80], breeding for increased seed yield in tetraploid red clover may therefore lead to unintentional selection for increased self-fertility. Self-fertility can result in higher seed yield, but it is likely that population vigor will decrease in the long run due to inbreeding depression. All depends on the severity of the inbreeding depression in tetraploid red clover, which has not been thoroughly investigated yet. No information is available on the effect of inbreeding on forage yield in red clover, but some information exists on seed yield. Laczynska-Hulewicz [89] found reductions of 23–26% in seed yield of tetraploid red clover inbred lines. Vleugels [80] found similar results: Genotypes that were the result of self-fertilization yielded on average 20.5% less seed than genotypes that were the result of cross-pollination. A related, unresolved issue is how exactly self-incompatibility works in tetraploid red clover [22]. Self-incompatible genotypes cannot be fertilized by their own pollen (pollen carrying two overlapping S-alleles), but it is unclear if they can be fertilized by pollen with one overlapping S-allele (e.g., pollen from a related genotype). If not, numerous genotypes are excluded from fertilizing such a genotype, especially in tetraploid populations harboring few S-alleles [80].

To conclude, embryo abortion is common in tetraploid red clover, but additional research is needed to compare the degree of abortion between diploids and tetraploids. Aberrations in male meiosis tend to occur more frequently in tetraploid red clover, and tend to be negatively associated with seed yield, although more research is needed to prove such associations. Pollen germination is also generally lower in tetraploid red clover. Finally, self-fertility seems relatively common in tetraploid red clover and can contribute to increased seed yield. Selection for seed yield in tetraploid red clover may lead to co-selection for self-fertility, yet more research is needed to fully unravel the consequences of inbreeding for forage yield and seed yield.

#### *4.4. Genomic Regions Associated with Seed Development and Seed Yield*

To date, only a few studies have investigated the genetics of seed yield in red clover. Herrmann [58] used a mapping population derived from a cross between two genotypes from the cultivars “Violetta” (high seed yield) and “Corvus” (poor seed yield) to identify genomic regions associated with seed yield. Thirty-eight QTLs related to eight seed yield components were identified and two genome regions containing four to five QTLs for different seed yield components were pinpointed as regions of interest for further QTL characterization. Recently, Kovi [52] used next-generation sequencing to analyze the transcriptome response during flowering and seed set in two tetraploid red clover genotypes with contrasting seed yield. They identified 34 differentially expressed genes related to pollination, flowering, and seed development, which can be considered as candidate genes for seed development in red clover [52]. The next step would be to perform loss-of-function analysis to understand the role of these candidate genes in seed development. Further efforts are therefore needed before genes affecting seed yield in red clover are identified and tools developed to exploit this information in practical breeding.

A second possible genetic factor is the effect of genetic diversity and heterozygosity on seed yield. The outcrossing nature of red clover suggests that increased heterozygosity leads to increased

fitness, both at the genotype level and the population level. Hence, populations with a broad genetic basis and a high degree of heterozygosity are expected to attain higher seed yields than populations with less genetic diversity. This might be particularly relevant in tetraploid populations, because the tetraploid red clover genepool has probably undergone a bottleneck as tetraploidization efforts are usually successful for a limited number of plants [6]. To our knowledge, no studies have assessed the link between genetic diversity and fitness or seed yield at the population level.

In conclusion, several QTL regions and candidate genes for seed yield and seed development have been identified, which opens perspectives for the development of molecular markers for seed yield. Furthermore, a reference genome is now available for red clover, opening perspectives for genomic selection [93].

#### *4.5. Implications for Breeding*

Keeping the recent findings in mind, breeders aiming to improve seed yield in diploid or tetraploid red clover should select plants with numerous flower heads and high seed yield numbers per flower head. Flowering traits such as corolla tube dimensions and flower color can be omitted as breeding targets, but if a population could be created with substantially increased nectar production, it is likely to attract more pollinators [35]. Identifying genotypes with deficient meiosis in order to exclude them from crossings may positively influence seed yield as well. However, analyzing meiosis in a breeding program is presumably too labor- and cost-intensive. Marker-assisted parentage analysis can increase genetic gains for seed yield by excluding progeny from low seed-yielding genotypes.

Regarding tetraploid red clover, breeders have at least two options to improve seed yield. Selection for seed yield can take place at the diploid level, followed by chromosome doubling and at least three generations of intercrossing [94]. In this regard, Dijkstra [59] recommended to select for plants with two-seeded pods on the diploid level, followed by tetraploidization as a means to increase seed yield in tetraploids. Improving seed yield through additive genetic variance is relatively straightforward in diploids [94]. Heterozygosity will be restored when sufficient generations of intercrossing are performed [88]. Alternatively, breeders can perform multiple generations of selection and crossing among existing tetraploid populations. In this way, heterozygosity remains high at all times. However, more generations of crossing and selection may be required to achieve the same additive genetic gain than at the diploid level, since four (instead of two) beneficial alleles need to be accumulated on each locus related to seed yield [80]. Moreover, selecting for seed yield in tetraploid red clover may increase the risk to co-select for self-fertility [80], the effects of which are likely not beneficial for seed yield and general performance in the long term. Nonetheless, self-fertile genotypes could be identified and their seeds discarded, so that only seeds from self-incompatible genotypes are transferred to the next breeding cycle. Detection would be possible with marker-assisted parentage analysis [56], or by isolating a few flowering stems in a bag with pollinators while leaving the remaining flowers to cross-pollinate. Today, it is difficult to state which of these two breeding methods is most suitable to increase seed yield in tetraploid red clover. Amdahl [9] found that populations made by crossing existing tetraploids displayed higher seed yields (8.1% and 21.4% higher in two experiments) than tetraploid populations created by chromosome doubling. However, the fact that the diploid ancestor cultivars were not deliberately selected for seed yield, renders it difficult to compare both breeding methods.

## **5. Conclusions and Perspectives**

Seed yield is a complex trait affected by various genetic, environmental, and agronomical aspects (Figure 1), some of which are not even discussed in this paper (e.g., disease resistance). However, all of the pieces of the puzzle together start to shed light on this complex process. The most important seed yield components are the number of flower heads per plant (in spaced plants) and the seed yield per flower head (in spaced plants and dense stands). Differences in seed yield between diploid and tetraploid red clover are mostly explained by the lower seed number per head and partly by the lower head number per plant at seed harvest.

The generally accepted statement that unsatisfactory seed yields in (tetraploid) red clover are mainly due to longer corolla tubes has been refuted by multiple studies. Corolla tube dimensions are not associated with seed yield, neither on a genotype basis nor in dense stands. Inadequate pollination can explain unsatisfactory seed yields in certain regions and years, e.g., where/when insufficient pollinators are present, but it cannot explain seed yield differences between genotypes, cultivars, or ploidy levels.

Recent studies seem to confirm that fertility problems may play an important role in seed yield, but more research is needed to confirm this. Meiotic aberrations tend to be more common in tetraploid red clover and in genotypes with low seed yield, with lower pollen germination as a consequence. A remarkable finding is the occurrence that high seed-yielding tetraploid genotypes are frequently self-compatible. Selection for seed yield in tetraploid red clover may perhaps lead to unintentional selection for self-fertility and a higher level of inbreeding. However, the consequences for inbreeding and the effects of inbreeding on seed yield and general performance in tetraploid red clover remain poorly understood, and should be the object of future research (currently underway at ILVO). Future research should also investigate how exactly self-incompatibility works in tetraploid red clover. An important question in this regard is whether self-incompatible genotypes can be fertilized by pollen with one overlapping S-allele (e.g., pollen from a related genotype).

So far, only few studies have investigated the genetics of seed yield in red clover. A transcriptome study revealed 34 candidate genes for seed development, which opens perspectives to develop markers for seed yield [52]. The recent construction of a reference genome for red clover is the first step towards genomic selection for seed yield [93]. Concerning breeding strategies, more research is needed to define which breeding method is the most suitable to increase seed yield in tetraploid red clover: Selection at the diploid level before tetraploidization, or selection at the tetraploid level. Elucidating the relation between genetic diversity and seed yield at the population level may help to uncover the most suitable breeding method.

## Abbreviation

Abbreviation	Full name
CTL	corolla tube length
CTD	corolla tube diameter
HN	head number per plant at seed harvest
SN/H	seed number per head at seed harvest
PGR	plant growth regulator
QTL	Quantitative trait locus

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