Seed Yield in Red Clover

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Red clover (*Trifolium pratense* L.) is a perennial forage legume which is valued for its yield, nitrogen fixation and forage quality. However, seed yield is often unsatisfactory in red clover. Seed production has been the objective of various studies with different approaches. This review paper summarizes and discusses recent results from various studies on seed yield and fertility in red clover, and opens perspectives for future research.

Trifolium pretense | 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][17][18][19][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.

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][25][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 stamina 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* ssp.) 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.

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