

Progress Made in Improving Red Clover (*Trifolium pratense* L.) Through Breeding

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ABSTRACT

Red clover (*Trifolium pratense* L.) is a major forage legume grown on approximately 4 million hectares worldwide. It has a long and varied history in agriculture. Active breeding efforts began at the end of the 19th century. Since this time, significant improvement in red clover cultivars has occurred. The major focus of breeding and accompanying research efforts has involved increasing persistence. Improved persistence has been achieved through general persistence selection, pathogen resistance breeding, and breeding for tolerances to other agronomic stresses. Dry matter yield has received less attention, but has nevertheless been improved. More recently, selection for adaptation and performance of red clover in a grass-legume mixture used especially in grazing has received attention. Genomic resources in red clover have matured with the availability of numerous genetic maps and publicly available molecular markers. Transformation techniques have been developed to allow creation of genetically modified plants. Numerous opportunities still exist to improve agronomic characteristics of red clover, particularly by continuing efforts to increase persistence and dry matter yield. In a world with expensive fossil fuel-based fertilizer, breeding to develop and maintain improved red clover will continue to remain important.

Keywords: dry matter yield, genetics, persistence, plant breeding

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INTRODUCTION

Red clover (*Trifolium pratense* L.) is a major forage legume grown on approximately 4 million ha worldwide (**Table 1**). It has a long and varied history in agriculture (Westgate and Hillman 1911; Fergus and Hollowell 1960; Smith *et al.* 1985; Undersander *et al.* 1990; Lacefield and Ball 1999). It is uncertain when red clover entered active cultivation; evidence of red clover in pastures dates to the Bronze Age in Europe (Hodgson *et al.* 1999; Rasmussen 2005). Berseem clover (*Trifolium alexandrinum* L.) was actively cultivated in ancient Egypt (Cagle 2001). Early herbalist manuscripts describe red clover and its medicinal purposes as early as at least Anglo-Saxon Britain (Cockayne 1961; Zeven 1991). Clover's agricultural benefits were extolled by Albert Magnus in the 12th century (Jessen 1982). In Medieval Spain Ibn al Awwam described the cultivation of a bright red flowered forage legume (with larger more "gracious" leaves than alfalfa) which he termed "burgundy grass (or forage)" and which later translators and authors associated with berseem clover (Awwam ~1200; Bolens 1981). Although it should be noted that the Burgundy region of Europe would likely be too cold for berseem clover, suggesting the possibility of a cultivated red clover originating from the Burgundy region during the medieval period. Definitive records of active red clover cultivation appear in the 16th century and are associated with intensification of agriculture (Blomeyer 1889; Merckenschlager 1934a, 1934b;

Table 1 Red clover (*Trifolium pratense* L.) seed production amounts. a – 2006/07 growing season estimates (unpublished), b – 2008 growing season (EU, 2009).

| Country or Region | Kg (Million) |
|--------------------------|-------------------|
| Argentina | 0.07 ^a |
| Canada | 1.23 ^a |
| European Union-27 | 5.30 ^b |
| New Zealand | 0.16 ^a |
| Norway | 0.10 ^a |
| Switzerland | 0.05 ^a |
| United States of America | 4.57 ^a |
| Total | 9.88 |

Lane 1980; Zeven 1991; Hopcroft 2003; Kjærsgaard 2003). By the 1800s almost all temperate agricultural regions in the world used red clover as an integral part of cropping rotations to improve soil and provide fodder. With the rise of widespread synthetic fertilizer use during the 20th century, a steep decline in red clover acreage was observed (Rhodes and Ortega 1996; Kjærsgaard 2003). This decline is evident in the United States during the 20th century from red clover seed production data (**Fig. 1**).

Today red clover is grown in pure stands or mixed with grass for hay, haylage, silage, or grazing (Undersander *et al.* 1990; Lacefield and Ball 1999). Red clover is known for rapid establishment, shade tolerance, and tolerance to low

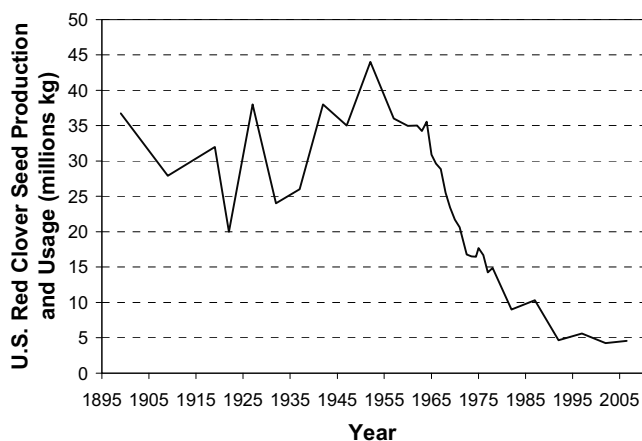


Fig. 1 United States red clover (*Trifolium pratense* L.) seed usage or production 1899-2007. Based on Taylor and Quesenberry (1996) and NASS (1899-2007).

pH, low fertility, and poorly drained soils (Fergus and Hollowell 1960; Smith *et al.* 1985; Undersander *et al.* 1990; Taylor *et al.* 1997). Red clover can serve a myriad of functions in agricultural rotations (Windham and Lawrence 1987; Knorek and Staton 1996; Sullivan 2003; Burdine *et al.* 2005; Chen *et al.* 2006; Quesenberry and Blount 2006).

Red clover is a perennial diploid ($2n = 14$). Almost all red clover varieties utilized in North America are diploid, while chromosome-doubled tetraploid red clover varieties are often bred and utilized in Europe in addition to diploid varieties (Taylor and Quesenberry 1996). Red clover has an estimated genome size of 435 Mb (Sato *et al.* 2005). Genomic work and infrastructure developed in other forage and model legumes species (i.e., *Lotus japonicus*, *Medicago sativa*, *Medicago truncatula*, and *Trifolium repens*) frequently are applicable to red clover genomics; including such activities as: cross species homologues gene discovery, molecular marker discovery, primer design for specific genomic regions (Choi *et al.* 2004). Taxonomically, red clover is a member of the *Leguminosae* in the genus *Trifolium* subgenus *Trifolium* sect. *Trifolium* (Zohary and Heller 1984; Ellison *et al.* 2006). The center of origin of red clover is believed to be in Eurasia along the Mediterranean Sea (Fergus and Hollowell 1960).

The dominant red clover breeding target has been increased persistence (i.e. longevity). Over a century of breeding for increased persistence has resulted in an expectation that modern varieties persist for 3 to 4 years; whereas in the past, red clover was sometimes considered a biannual (Smith 2000; Riday and Krohn 2010a; **Table 2**). Gains in persistence have been achieved by generally focusing on specific plant pathogens and stresses (Taylor 2008a).

BREEDING FOR PATHOGEN RESISTANCE

Red clover mortality is most frequently observed in conjunction with deteriorating crowns and roots. *Fusarium* spp. root rot are often found in these situations. Winter-injury and mechanical damage of the crown increases pathogen incidence. Managing stands for good fall growth and minimizing mechanical plant damage are recommended cultural practices to address *Fusarium* (Undersander *et al.* 1990). Breeding efforts to improve resistance in cultivars have been applied in many breeding programs. However, selection for *Fusarium* resistance in red clover has been difficult (Venuto *et al.* 1999) due to the multitude of species and isolates that infect red clover and the complex genetics of resistance (Velde 1980) and fungal by environment interactions (Venuto *et al.* 1996). Greenhouse (Nedelnik 1986, 1989; Venuto *et al.* 1999) and field (Rufelt 1985; Nedelnik 1988; and Leath *et al.* 1989) inoculation techniques have been developed and increased resistance achieved in greenhouse-grown plants (Anderson and Kristiansson 1989; Pokorný *et al.* 2003). Under field conditions, Andersson (1985) observed improved *Fusarium* resistance after three cycles of selection; Coulman and Lambert (1995) observed no progress after one cycle of selection; while Venuto (1999) observed slow progress. It has been noted that local cultivars are usually more adapted to local *Fusarium* species and races, indicating that general adaptation breeding for *Fusarium* resistance may be difficult (Chapurin *et al.* 1988, Kovacikova *et al.* 1991). Heritability estimates are 0.06-0.37, likely necessitating progeny testing and multi-year evaluation to achieve breeding progress (Coulman and Lambert 1995, Venuto *et al.* 1999; Gaue and Ingwersen 2003). Nedelnik (1992) concluded that greenhouse-selected resistance to *Fusarium* was not related to field mortality, which was caused by complex biotic and abiotic factors not related to the fungal pathogen. However, Gaue and Ingwersen (2003) felt that greenhouse and field selections did concur, but that field studies required more care and resources to conduct. Additionally, Pederson *et al.* (1980) and Taylor (2008) observed reduced root size with increased *Fusarium* resistance, potentially an adverse correlation. However, increased winterhardiness is associated with increased *Fusarium* resistance (Smurygin and Vinnik 1991).

Sclerotinia trifoliorum Eriks. is another major root pathogen in red clover. *Sclerotinia* is the major fungal pathogen afflicting red clover in Europe, Japan, and South America; although it is less of a concern in North America (Undersander *et al.* 1990; Rhodes and Ortega 1996; Pokorný *et al.* 2003; Abberton and Marshall 2005; Taylor 2008a). Öhberg (2008) offers an excellent review of *Sclerotinia*. Screening techniques for *Sclerotinia* have been developed based on mycelium (Frandsen 1946; Dijkstra J. 1964; Dixon and Doodson 1974), but the reliability of these techniques has been questioned (Marum *et al.* 1994; Taylor and Quesenberry 1996). This led to the development of newer

Table 2 Estimates of genetic gain in red clover breeding. † Woodfield and Brummer (2001), ‡ Data from Six trials planted 1986 to 1991 in Wisconsin, USA. (Smith *et al.* 1987-1994; Riday and Krohn 2010a), § Data from 1 Rotational Grazed red clover – tall fescue mixture trial established 2004 in Wisconsin, USA (Riday *et al.* 2007), ¶ Unpublished results from (Riday *et al.* 2007), a – released 1953 (Hollowell 1961); b – released 1973 (Smith *et al.* 1973); c – released 1987 (Smith *et al.* 1994); and d – unreleased experimental first tested in 1992 (Smith 2000).

| Trait | Benchmark variety | Improved varieties | Genetic gain (% yr ⁻¹) |
|---|-----------------------|--|------------------------------------|
| DM yield† | G. Turoa | G. Hamua, Pawera | 0.43 |
| DM yield† | G. Hamua | G. Colenso | 0.21 |
| DM yield† | G. Pawera (4x) | G27 (4x) | 1.39 |
| 1 st post-seeding year DM yield‡ | Lakeland ^a | Arlington ^b , Marathon ^c | 0.41 |
| 2 nd post-seeding year DM yield‡ | Lakeland | Arlington, Marathon | 0.55 |
| 3 rd post-seeding year DM yield‡ | Lakeland | Arlington, Marathon | 0.95 |
| Trial DM yield‡ | Lakeland | Arlington, Marathon | 0.60 |
| Establishment survival§ | Lakeland | Arlington, Marathon, C328 ^d | 0.94 |
| 12-month survival§ | Lakeland | Arlington, Marathon, C328 | 1.94 |
| 24-month survival§ | Lakeland | Arlington, Marathon, C328 | 1.43 |
| 36-month survival¶ | Lakeland | Arlington, Marathon, C328 | 2.78 |
| 48-month survival¶ | Lakeland | Arlington, Marathon, C328 | 1.32 |

ascospore inoculation techniques (Delclos *et al.* 1997). Populations with resistance to the pathogen have been reported (Valleau 1933; Arseniuk 1989). RAPD markers have been reported associated with resistance to the disease (Page *et al.* 1997). Klimenko *et al.* (2010) recently mapped QTL associated with *Sclerotinia* and *Fusarium* resistance, along with QTL associated with winterhardiness.

Northern anthracnose (*Kabatiella caulivora* [Kirch.] Karak.) is a foliar fungal pathogen occurring in cooler climates (Undersander *et al.* 1990; Taylor and Quesenberry 1996). Breeding in North America has resulted in resistant cultivars (Smith and Maxwell 1973; Smith 1994). Southern anthracnose (*Colletotrichum trifolii* B. and E.) occurs in warmer climates, resistant cultivars to southern anthracnose were created in the 1950's in North America (Taylor 2008a). Through these breeding efforts both pathogens became less of a problem, with most improved cultivars containing resistance. Recently, with continued global climate change, southern anthracnose has shifted north (Boller *et al.* 1998). This shift has led to new efforts to breed southern anthracnose resistance in Europe (Schubiger *et al.* 2003). Other pathogens that have received some attention include powdery mildew (*Erysiphe polygoni* D.C.), for which some resistant cultivars are available (Taylor and Ghabrial 1995; Taylor 2008a, 2008b), leaf spot or target spot (*stemphium sacrcinaeforme* (Cov.) Wiltshire) (Smith and Kretschmer 1989), and *Aphanomyces euteichers* Drechs (Tofte *et al.* 1991). Breeding for clover rust (*Uromyces trifolii* var. *fallens*) and another leaf spot (*Pseudopeziza trifolii*) has not been initiated (Pokorný *et al.* 2003; Taylor 2008a). Among viruses, only the bean yellow mosaic virus (BYMV) is considered agronomically important (Taylor 2008a), and resistance breeding has been successful for this virus (Leath *et al.* 1987b; Smith 1994; Taylor and Ghabrial 1995; Pokorný *et al.* 1995; Pokorný and Jakešová 2003).

Resistance breeding for four nematode pests has been conducted: root-knot nematode (*Meloidogyne* spp.) (Hussey and Barker 1973; Quesenberry *et al.* 1986, 1989, 1993, 2005); root lesion nematode (*Pratylenchus penetrans* (Cobb) Chitwood Oteifa) (Willis *et al.* 1971; Kimpinski *et al.* 1984; Papadopoulos *et al.* 2002, 2003); clover cyst nematode (*Heterodera trifolii* (Goffart) Oostenbrink) (Norton and Isley 1967; Leath *et al.* 1985; Leath *et al.* 1987a); and stem nematode (*Ditylenchus dipsaci* (Kühn) Filipjev) (Bingefors 1952). For the latter stem nematode, extensive breeding in Europe has been conducted during the 20th century (Taylor and Quesenberry 1996), resulting in most commercial varieties being resistant to this pest (Bingefors 1985; Pokorný *et al.* 2003).

Other pests that afflict red clover include: pea aphid (*Acyrtosiphon pisum* Harris), root borer (*Hylastinus obscurus* Marsham), clover leaf weevil (*Hypera nigrirostris* Fabr.), red clover weevil or clover head weevil (*Phytonomus meles* Fabr. Or *Hypera meles* Fabr.), clover root curculio (*Sitona hispidula* F.), and stem weevils (*Apion* spp.) (Taylor and Quesenberry 1996). Of special note in North America is the potato leafhopper (*Empoasca fabae* Harris); most North American red clover germplasm is resistant to the potato leafhopper through naturalization. All non-North American germplasms suffer tremendously during annual summer onslaughts of the pest, making it almost impossible to grow these latter germplasms and creating a barrier for breeder utilization of non-naturalized germplasm in North America. The resistance of North American germplasm to the potato leafhopper has been long associated with its more abundant plant pubescence in comparison to germplasm from other regions (Hollowell *et al.* 1927; Monteith and Hollowell 1929; Pieters and Hollowell 1937; Simmons *et al.* 1984; Choo *et al.* 1987; Kusmiyata *et al.* 1996; Taylor and Collins 2003).

An alternative approach to increasing persistence of red clover has been to focus on morphological traits or general selection for persistence in a field setting. Narrow sense heritabilities for persistence on an individual plant basis have been estimated at 0.171 and 0.142 (Anderson *et al.*

1974; and Cornelius *et al.* 1977) and 0.327 on a halfsib family mean basis (Riday, unpublished; 6 plant plots × 2 replicates × 7 environments). The possibility of such selection was demonstrated in the variety 'Hokuseki' which was bred based on third year forage yield using maternal-line selection and showed improved persistence over its derived population (Yamaguchi *et al.* 2000; Okumura 2005). In Canada 'AC Endure' and 'AC Christie' were developed for increased persistence using recurrent mass selection (Christie *et al.* 1998; Martin *et al.* 1999). Herrmann *et al.* (2008) have identified several QTL associated with field measured persistence.

Another area of interest has been selection for "adventitious" or "fibrous" roots to increase persistence (Montpetit and Coulman 1991; Smith 1989; McLean and Nowak 1998). Field observations show that plants that have survived a long time in the field tended to have more fibrous roots (i.e. lack of central taproot) (Taylor *et al.* 1962; Cressman 1967; Ryle *et al.* 1981; Kendall and Stringer 1985; Riday, unpublished). Researchers found that selection for root type was possible (Montpetit and Coulman 1991; Smith 1989; McLean and Nowak 1997). However, direct selection for root type did not necessarily lead to improved persistence (Smith 1989; McLean and Nowak 1998), but selections out of old stands, regardless of root type, did lead to an increased frequency of fibrous root-type genotypes (Smith 1989).

BREEDING FOR INCREASED DRY MATTER YIELD

In addition to persistence, dry matter (DM) yield remains a key breeding target in red clover. Steady gains of between 0.21 and 1.39% per year for DM yield have been observed (**Table 2**). Visual scores of yield are very effective at estimating yield for selection purposes. Riday (2009) demonstrated that on a halfsib progeny mean basis simple visual scores of space planted red clover plants had correlations higher than 0.9. In many cases, however, gains in DM yield have been subordinate to efforts focused on increasing persistence, which show gains of 1.32 to 2.78% per year (**Table 2**; Smith 2000; Riday and Krohn 2010a). Interestingly, breeding for increased persistence has indirectly led to better seedling establishment (Riday *et al.* 2007; **Table 2**).

Comprehensive reviews on red clover field breeding methods were given by Fergus and Hollowell (1960), Taylor and Smith (1979), and Taylor and Quesenberry (1996). Red clover is somewhat more difficult to clone compared to other forage legumes. Due to its shorter life span, red clover is more difficult to maintain over longer periods of years, which is necessary for certain breeding strategies. These factors have led to a few basic breeding strategies that are commonly used in red clover. Mass selection has been used extensively to select for pest resistance and persistence in red clover and has been often accomplished recurrently (i.e. recurrent phenotypic selection [Fehr 1991]). Mass selection is the simplest form of selection but is less effective with traits highly subject to macro and micro-environmental variation (Fehr 1991). Various forms of halfsib selection are frequently used and are referred to as: 'line selection', 'maternal-line selection' (Okumura 2005), 'recurrent phenotypic selection' (in this case refers to individual selection in cycle 0, as in mass selection, with replicated or unreplicated evaluation of halfsib families in the next cycle of selection), 'geno-phenotypic selection' (i.e. selection among and within best halfsib families [Taylor and Quesenberry 1996]), and 'progeny testing'.

Almost no studies comparing alternate breeding methods exist in red clover (Taylor 1987). Many studies have reported heritabilities for various traits using the different breeding methods (Taylor and Smith 1979). One breeding method comparison in red clover has been published. Ledda *et al.* (2000) compared 2 cycles of mass selection for DM yield to one cycle of selection among 50 halfsib families. Two populations were developed using halfsib selections: one based on intermating the original parents of selected

half-sib families (i.e. “progeny testing”), and the other by bulking equal quantities of selected half-sib remnant seed. Selection gains compared to the original population were 7.7% (two cycles mass selection, selection intensity 10% during each cycle), 9.2% (once cycle half-sib selection, remnant seed intermated, selection intensity 20%), and 25.3% (once cycle half-sib selection, original parents intermated, selection intensity 20%).

Proposals to capture non-additive genetic effects and increase DM yield through hybrids have not as yet produced a commercial cultivar (Townsend and Taylor 1985). Red clover has a strong gametophytic self-incompatibility system and like many types of forage does not tolerate inbreeding well (Riday and Krohn 2010b). Townsend and Taylor (1985) offer a review of research attempts to create red clover hybrids with self-compatible inbred lines. Much of the research was carried out in the 1960s and 1970s. It appears that the costs required to develop and identify superior inbred lines for use in commercially viable red clover breeding programs were prohibitive, as none have been developed. Recently, with renewed interest in population hybrids or semi-hybrid populations in forages (Brummer 1999), a new proposal involving the restriction of S-alleles in red clover populations (i.e. three S-alleles per population) followed by the creation of population hybrids among these restricted populations potentially offers a less expensive hybrid seed production method that does not require inbreeding (Riday and Krohn 2010a).

Tetraploid red clover has been used as a means to increase DM yield. Commercial tetraploid red clover varieties have been successfully developed in Europe starting in Sweden in 1957 (Öhberg 2008). Since then, tetraploid red clover varieties have been developed throughout Europe and Japan (Boller *et al.* 2001; Gaue and Ingwersen 2003; Okumura 2005). A few naturally occurring tetraploid red clover populations in Turkey have been noted and studied (Büyükkartal 2003, 2008). A review of the early Swedish tetraploid work is given by Sjödin and Ellerström (1986). Tetraploid red clover has been shown to have increased plant size (Levan 1945; Jonsson 1985; Sjödin and Ellerström 1986; Tomaszewski 1988). Additionally, increased disease resistance and persistence in tetraploids in comparison to diploids has been observed in some cases (Boller *et al.* 2003) but not in others (Öhberg *et al.* 2005). The major disadvantage of tetraploids is lower seed yield (Sjödin and Ellerström 1986; Taylor and Quesenberry 1996). Seed yield deficits of 28% continue to be reported (Zuk-Gotaszewska *et al.* 2006). Breeding efforts to increase and understand seed formation and seed yield continue today (Malengier and Baert 2007; Büyükkartal 2008). Although seed yield in tetraploids has received much attention, only a few breeding studies from Russia have reported to improving seed yield in diploids (Smolikova *et al.* 1987; Voronchikhina *et al.* 1987; Novoselova *et al.* 1988; Quesenberry and Taylor 1996). Recently a mapping study from Switzerland identified some seed yield QTLs (Hermann *et al.* 2006). Despite the paucity of reports on seed yield breeding, this trait is of great interest in cultivar development and particularly commercialization and is sometimes reported in cultivar registrations (Yamaguchi 2000).

BREEDING FOR MIXTURES IN PASTURES

In recent years, as red clover usage has stabilized (Fig. 1), much of the remaining acreage is grown in grass-legume mixtures subjected to grazing. This has led to an interest in developing grazing tolerant red clover varieties. One of the first grazing tolerant red clover varieties developed was ‘Astred’ (Smith 1992; Smith and Bishop 1993, 1998), which was developed from prostrate stoloniferous root forming red clover germplasm collected from Portugal. Since then, additional cultivars have been released/marketed: ‘Crossway’ and ‘G. Broadway’ (Rumball *et al.* 2003 – New Zealand) (Spanish prostrate germplasm base); ‘G. Sensation’ (Calydon *et al.* 2003 – New Zealand) (Swiss germ-

plasm base); ‘RedlandGraze’ series (ABI ~ 1999 – know Forage Genetics, USA) (‘Redland’ – “Illinois synthetic” germplasm base); ‘Cinnamon Plus’ (FFR ~ 1999, USA). In cooler climates in North America, rotational grazing is practiced on pastures, as opposed to set-stocking, which occurs in warmer climates of North America. A study by Riday *et al.* (2007) showed that more recently released varieties were more persistent under rotational grazing than older varieties (Table 2). No differences were observed between grazing type varieties and other newer red clover varieties, although newer germplasm from outside North America, including ‘Astred’, did not persist well.

Breeding for red clover varieties specifically adapted to a grass-legume mixture has come to the fore in the last decade (Smith 2000; Real *et al.* 2000; Iepema 2004; Riday *et al.* 2007). Some red clover breeders are selecting plants out of nurseries with a grass companion; this is already common in white clover and alfalfa (Woodfield and Brummer 2001; Riday 2009). In addition, Riday (2008) examined heritabilities for establishing red clover lines in permanent pasture using broadcast seeding as a precursor to selecting for this trait. In an opposite fashion, ‘Natsuyu’ was developed in Japan to be less competitive with timothy as a companion grass (Isobe *et al.* 2002). Selection for 2,4-D tolerance has been conducted; this would be a favorable trait to have in red clover in order to better manage weeds in grass-legume pastures (Taylor *et al.* 1989a, 1989b).

To better integrate red clover into sheep grazing systems, breeding against anti-quality traits has been conducted. Red clover phytoestrogens are detrimental to sheep (Kramer *et al.* 1996; Bush *et al.* 2007). Reduced formononetin (i.e. the major red clover isoflavone or phytoestrogen related compound) red clover varieties have been successfully bred to address this (McDonald *et al.* 1994; Rumball *et al.* 2003). An interesting side note to this effort is the recent interest from a human medicinal perspective to increase isoflavone levels in red clover for the nutraceutical industry; this effort has led to the development of some high formononetin red clover varieties and germplasm (Rumball *et al.* 1997, 2005; Papadopoulos *et al.* 2006; Tsao *et al.* 2006).

GERMPLASM DIVERSITY AND MOLECULAR GENETICS

A vast array of red clover germplasm and cultivars exist. Recently, attempts have been made to characterize this diversity using molecular markers. Genetic diversity studies have been carried out with: isozyme markers (Hickey *et al.* 1991; Kongkiatngam *et al.* 1995; Hagen and Hamerick 1998; Yu *et al.* 2001; Semerikov *et al.* 2002; Mosjidis *et al.* 2004; Malaviya *et al.* 2005; Mosjidis and Klingerler 2006); restriction digests (Milligan 1991); RFLP (Nelke *et al.* 1993); RAPD (Kongkiatngam *et al.* 1995, 1996; Campos-de-Quiroz and Ortega-Klose 2001; Ulloa *et al.* 2003; Greene *et al.* 2004); AFLP (Kölliker *et al.* 2003; Hermann *et al.* 2005); and SSR (Dias *et al.* 2008). All studies found that the majority of neutral molecular genetic variation was contained within populations rather than between. All studies looking at local wild populations or landraces versus outside germplasms could differentiate the groups (Campos-de-Quiroz and Ortega-Klose 2001; Semerikov *et al.* 2002; Kölliker *et al.* 2003; Ulloa *et al.* 2003; Hermann *et al.* 2005). Greene *et al.* (2004) observed that among wild populations, only those found in isolated areas could be geographically differentiated using molecular markers. However, global studies of molecular genetic diversity failed to produce coherent classifications based on morphology, geography, or known breeding histories (Kongkiatngam *et al.* 1996; Yu *et al.* 2001; Mosjidis and Klinger 2006; Dias 2008). The role of this work in plant breeding has so far mainly promoted existing efforts to improve unique landraces for development into cultivars (Boller 2000; Helgadottir *et al.* 2000; Boller *et al.* 2004). Attempts to bring diversity from other clover species into red clover

have been limited; this work is fully reviewed by Taylor and Quesenberry (1996) and Abberton (2007).

Molecular maps have been developed in red clover using RFLP (Isobe *et al.* 2003), AFLP (Herrmann *et al.* 2006, 2008), and SSR (Sato *et al.* 2005; Kölliker *et al.* 2005; Herrmann *et al.* 2008; Klimenko *et al.* 2010). Recently Isobe *et al.* (2009) published a consensus map for red clover. QTL mapping was conducted in the Herrmann *et al.* map for seed yield and persistence traits. Flower color was mapped on the Isobe *et al.* (2003) linkage map. The Sato *et al.* (2005) SSR map showed potential macro-colinearity with a white clover map (Zhang *et al.* 2007). Page *et al.* (1997) used bulk segregant analysis in an attempt to generate RAPD markers associated with *Sclerotinia* resistance. Klimenko *et al.* (2010) mapped QTL for *Sclerotinia* and *Fusarium* susceptibility and winterhardiness. Systems integrating red clover molecular markers into red clover breeding programs have been demonstrated (Riday 2007, 2010a, 2010b, 2010c).

Agrobacterium-mediated transformation techniques have been developed for red clover (Quesenberry *et al.* 1996; Mouradov *et al.* 2006; Sullivan and Quesenberry 2006). Red clover capable of regenerating from callus tissue is necessary to accomplish transformation; such germplasm has been developed (Quesenberry and Smith 1993; Smith and Quesenberry 1995). Red clover tissue culture methodologies are extensively reviewed by Taylor and Quesenberry (1996) including methodologies such as: media development, meristem culture, callus induction and plant regeneration, somatic embryogenesis and somaclonal variation, protoplast culture, and ovule and embryo culture. Thus far, red clover transformation is being used to identify red clover genes involved with proteolytic inhibition for potential transfer in alfalfa (Sullivan *et al.* 2004; Sullivan and Hatfield 2006; Sullivan 2009; Sullivan and Zarnowski 2010). Natural genetic variability of red clover for proteolytic inhibition levels had been determined in an earlier study (Broderick *et al.* 1994).

CONCLUSIONS

Although red clover usage has declined since the 1950s, it still remains a major forage crop around the world. A century of red clover breeding has led to clear gains in the agronomic performance of the species. Steady breeding for increased persistence and DM yield still have merit, as evidenced by past improvements. Current efforts world wide are continuing this steady improvement, as well as addressing the many specific uses of red clover. With cheaper molecular genetic costs and improved genomic infrastructure, increased integration of these techniques into cultivar development programs may soon occur.

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