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ORIGINAL RESEARCH

Validation of DNA marker-assisted selection for forage biomass productivity under deficit irrigation in alfalfa

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Abstract

Drought and limited irrigation resources threaten agricultural sustainability in many regions of the world. Application of genomic-based breeding strategies may benefit crop variety development for these environments. Here, we provide a first report on the effect of deploying DNA marker-assisted selection (MAS) for the drought resilience quantitative trait in alfalfa (*Medicago sativa* L.). The goals of this study were to validate the effect of several quantitative trait loci (QTL) associated with alfalfa forage and crown–root (CR) biomass during drought and to determine their potential to improve forage yield of elite germplasm under water-limited conditions. Marker assisted selection was employed to introgress favorable or unfavorable DNA marker alleles affiliated with 10 biomass QTL into three elite backgrounds. Thirty-two populations were developed and evaluated for forage productivity over 3 yr under continuous deficit irrigation management in New Mexico, USA. Significant yield differences (ranging from −13 to 26%) were detected among some MAS-derived populations in all three elite backgrounds. Application of QTL MAS generally resulted in expected phenotypic responses within an elite genetic background that was similar to that in which the QTL were originally identified. However, relative performance of the populations varied substantially across the three genetic backgrounds. These outcomes indicate that QTL MAS can significantly affect forage productivity of elite alfalfa germplasm in drought-stressed environments. However, if biomass QTL are detected in donor germplasm that is genetically dissimilar to targeted elite populations, characterization of donor alleles may be warranted within elite backgrounds of interest to confirm their phenotypic effects prior to implementing MAS-based breeding.

Abbreviations: ANTE(1), first-order antedependence; $CHBC_1$, Chilean first-generation backcross; CR, crown–root; CS, compound symmetry; HCR, high-crown-root; HS, high-shoot; IM, interval mapping; LCR, low-crown-root; LG, linkage group; LRWC, leaf relative water content; LS, low-shoot; MAS, marker-assisted selection; NMBM, 'NuMex Bill Melton'; QTL, quantitative trait loci; SNP, single-nucleotide polymorphism; SSR, simple sequence repeat; $WFBC_1$, Wisfal first-generation backcross.

1 | INTRODUCTION

Alfalfa (*Medicago sativa* L.) is an autotetraploid ($2n = 4x = 32$) perennial forage legume that is grown worldwide for hay, pasture, and silage (Li & Brummer, 2012; Yuegao & Cash, 2009). Many of these production environments experience some degree of water stress on a regular basis (FAO, 2021). In the United States, for instance, 70% of the alfalfa acreage resides in the Great Plains and the western regions (USDA–National Agricultural Statistics Service, 2021). A majority of this acreage relies on irrigation for successful hay production (Mubako & Lant, 2013; USDA–National Agricultural Statistics Service, 2018). However, frequent occurrence of drought and rapidly diminishing water resources for irrigation in this area regularly limit alfalfa forage yield (Orloff & Putnam, 2015; National Drought Mitigation Center, 2021).

Improving crop productivity in drought-prone environments is a major goal of plant breeding (Blum, 2005, 2009; Cattivelli et al., 2008; Gudys et al., 2018; Mir et al., 2012; Nepolean et al., 2018; Reynolds & Tuberosa, 2008; Sinha et al., 2021). In this regard, conventional breeding approaches have been used to develop alfalfa cultivars and germplasm with improved productivity under reduced irrigation allotments (Melton et al., 1989b; Ray et al., 1999c, 2012). However, progress has been slow because of (a) long breeding cycles affiliated with alfalfa's perennial nature and requirement of multiple-year evaluations, (b) complexities associated with polysomic inheritance and moderate to low trait heritability in elite populations, and (c) genotype × environment interactions (Li & Brummer, 2012; Ray et al., 1999a, 1999b, 2015). Opportunities to accelerate improvement of alfalfa drought resilience may also be realized through the identification of DNA marker alleles located at or near quantitative trait loci (QTL) associated with drought resistance traits and forage productivity during drought stress (Ray et al., 2015; Santantonio et al., 2019; Yu, 2017; Zhang et al., 2015). DNA marker-assisted breeding using conventional QTL introgression methods or genomic selection strategies (Li & Brummer, 2012; Li et al., 2015; Annicchiarico et al., 2015) can subsequently be used to increase the frequency of targeted favorable alleles in elite populations. Selection against undesirable alleles may also be practiced to benefit traits of interest. Although markers or loci associated with drought tolerance have been identified in alfalfa using traditional QTL mapping (Ray et al., 2015) and advanced genome-wide association studies (Zhang et al., 2015; Yu, 2017), we are not aware of any reports describing QTL marker-assisted selection (MAS) for this trait in alfalfa.

In a previous report by Ray et al. (2015), 25 QTL associated with alfalfa forage productivity under deficit irrigation management were identified. That germplasm was developed by intermating two unimproved autotetraploid genotypes, CH28 (*M. sativa* L. subsp. *sativa* var. Chilean; high yielding and low water-use efficiency) and WF1 (*M. sativa* L. subsp. *falcata* var. Wisfal; low yielding and high water-use efficiency) (Bingham, 1993; Ray et al., 2004). A single F_1 progeny (CW192) was then backcrossed to both parents to produce the Chilean first-generation backcross ($CHBC_1$) and Wisfal first-generation backcross ($WFBC_1$) mapping populations. Both populations were genotyped with 600 simple sequence repeat (SSR) and single-nucleotide polymorphism (SNP) markers that segregated as single-dose alleles (Sledge et al., 2005). Half-sib families from both populations were evaluated for forage yield in seeded plots in seven water-stressed environments. Interval mapping in the $CHBC_1$ and $WFBC_1$ populations identified 10 favorable and 15 unfavorable shoot biomass QTL. Six additional QTL affiliated with crown–root (CR) biomass were subsequently identified in the $CHBC_1$ population (unpublished data, 2009). Ray et al. (2015) hypothesized that the biomass QTL identified in their research might be useful for improving forage yield of elite populations in water-limited environments. In the current study, we tested that hypothesis. Specifically, we employed MAS for favorable or unfavorable DNA marker alleles at 10 of those biomass QTL and evaluated the potential of those alleles to alter forage productivity in progressively elite genetic backgrounds over 3 yr under deficit irrigation management.

Core Ideas

- Previous studies identified QTL associated with alfalfa biomass during drought.
- Effects for selected QTL marker alleles were validated in elite families related to donor germplasm.
- Confirmation of QTL marker allele effects is warranted if donor and target germplasm are unrelated.
- Genomic-based breeding strategies can benefit alfalfa productivity during drought.

2 | MATERIALS AND METHODS

2.1 | Semi-elite C_0 base population development and genotyping

Previously, Ray et al. (2015) conducted interval mapping (IM) in two alfalfa first-generation backcross mapping populations, designated $CHBC_1$ and $WFBC_1$, to identify forage biomass QTL in seven water-stressed environments during 2005 to 2007. Among the three parents of the two BC_1 populations, 11 shoot biomass QTL were identified in the CH28 and WF1 recurrent parents, and 14 QTL were identified in their F_1 hybrid, CW192. When the above study was terminated in

2007, data for CR biomass were also obtained by excavating all plots of the $CHBC_1$ population to a 25-cm soil depth and weighing their plant crowns and attached roots (Supplemental Materials and Methods). Results affiliated with the CR biomass trait have not been reported previously. Those outcomes will be described here based upon phenotypic data and IM analysis approaches defined by Ray et al. (2015). Importantly, the IM analysis detected six significant CR biomass QTL in $CHBC_1$. Collectively, the IM results indicated that CW192 possessed eight favorable and six unfavorable shoot biomass QTL and two favorable and two unfavorable CR biomass QTL. To validate the effect of some of the above biomass QTL in more elite genetic backgrounds, the CW192 F_1 hybrid plant was selected to serve as a QTL donor in the current study.

Introgression of single-dose biomass QTL marker alleles into more elite genetic backgrounds began by mating a single plant (Malone2) from the 'Malone' cultivar (*M. sativa* L. subsp. *sativa*, Melton et al., 1989a) with the CW192 plant. A semi-elite C_0 population (50% elite background) of 200 full-sib progeny was produced (Figure 1). Both parents were genotyped as described by Sledge et al. (2005) using 27 SSR markers previously reported to be associated with either high-shoot (HS), low-shoot (LS), high-crown–root (HCR), or low-crown–root (LCR) biomass production (Ray et al., 2015, and this paper). In many cases, the Malone2 parent possessed marker alleles that appeared similar in size to alleles observed in CW192 that were the target of MAS. This was particularly common for CW192 alleles derived from the CH28 (subsp. *sativa*) parent. When such circumstances occurred, alternative marker alleles that resided within the genome-wide or linkage group (LG) logarithm of odds (1 LOD) interval for the targeted QTL were evaluated for their ability to clearly discriminate between Malone2 and CW192 alleles. Ultimately, we were able to identify 10 informative marker alleles (one donated from CH28 and nine donated from WF1) for genotyping the semi-elite full-sib C_0 population (Table 1; Supplemental Table S1). These markers were associated with six different shoot biomass QTL and four different CR biomass QTL. All targeted QTL markers were present as single-dose alleles in the CW192 parent (Ray et al., 2015). According to tetrasomic inheritance models, each marker allele was expected to segregate 1:1 in the CW192 × Malone2 semi-elite full-sib C_0 population.

2.2 | Development of C_0Syn1 and MAS Syn1 populations

Based on the genotyping results of the C_0 population, QTL MAS was imposed. Limited marker coverage in the targeted genome regions precluded the use of flanking markers for MAS. Ten semi-elite MAS genotype groups of plants, comprised of 18–35 individuals each, were selected from the C_0 population and designated as HS1+ and HS1−, HS3+ and HS3−, LS2+ and LS2−, HCR2+ and HCR2−, and LCR2+ and LCR2−. The HS1+ and HS1− MAS genotype groups respectively represented selection for (+) or against (−) the only CH28-derived HS biomass marker allele (HS1) evaluated in this study (i.e., marker AW86 located on LG 1C; Table 1). Twenty-four C_0 plants that possessed HS1 were chosen for the HS1+ genotype group, while 19 individuals that did not possess this allele were chosen for the HS1− genotype group. The remaining nine marker alleles evaluated in this study were derived from the WF1 parent. These included three HS biomass alleles (HS3) and two alleles each that were affiliated with LS (LS2), HCR (HCR2), or LCR (LCR2) biomass. We hypothesized that imposing MAS for >1 marker allele might enhance phenotypic effect. Hence, MAS was simultaneously directed at all targeted WF1-derived marker alleles affiliated with a given biomass phenotype. For example, the HS3+ and HS3− MAS genotype groups represented simultaneous selection for or against three HS biomass marker alleles (AL22, BE137, and BE140) located on LG1A, LG6A, and LG8A, respectively. Twenty-seven C_0 plants, each of which possessed all three favorable QTL marker alleles, were assigned to the HS3+ genotype group. Twenty-seven other individuals that did not possess any of these marker alleles were assigned to the HS3− genotype group. A similar approach was used to establish the LS2+, LS2−, HCR2+, HCR2−, LCR2+, and LCR2− MAS genotype groups, which consisted of 21, 21, 25, 18, 20, and 35 C_0 plants, respectively. These 10 semi-elite MAS genotype groups of plants were randomly intercrossed by hand in a greenhouse using multiple rounds of reciprocal chain-mating within their respective groups to produce 10 semi-elite MAS Syn1 populations. All 200 C_0 plants were also intermated in a similar fashion to generate a semi-elite C_0Syn1 reference population. A balanced composite of seed from each plant involved in each cross was used to represent the 11 Syn1 populations for subsequent forage yield phenotyping (Figure 1, Table 2).

2.3 | Development of Elite(MAS+), Elite(C_0) reference, and elite check populations

The semi-elite MAS plants assigned to the HS1+, HS3+, LS2+, HCR2+, and LCR2+ genotype groups (collectively referred to as MAS+) and all semi-elite C_0 plants were randomly and reciprocately mated by hand to 60 plants each from three diverse elite alfalfa populations. The same 60 plants were used for all crosses. For each reciprocal single-cross generated between any two plants, a clean pollen applicator card was used to maximize interpopulation mating and to prevent intrapopulation mating. The three elite populations included the cultivars Malone (Melton et al., 1989a) and NuMex Bill

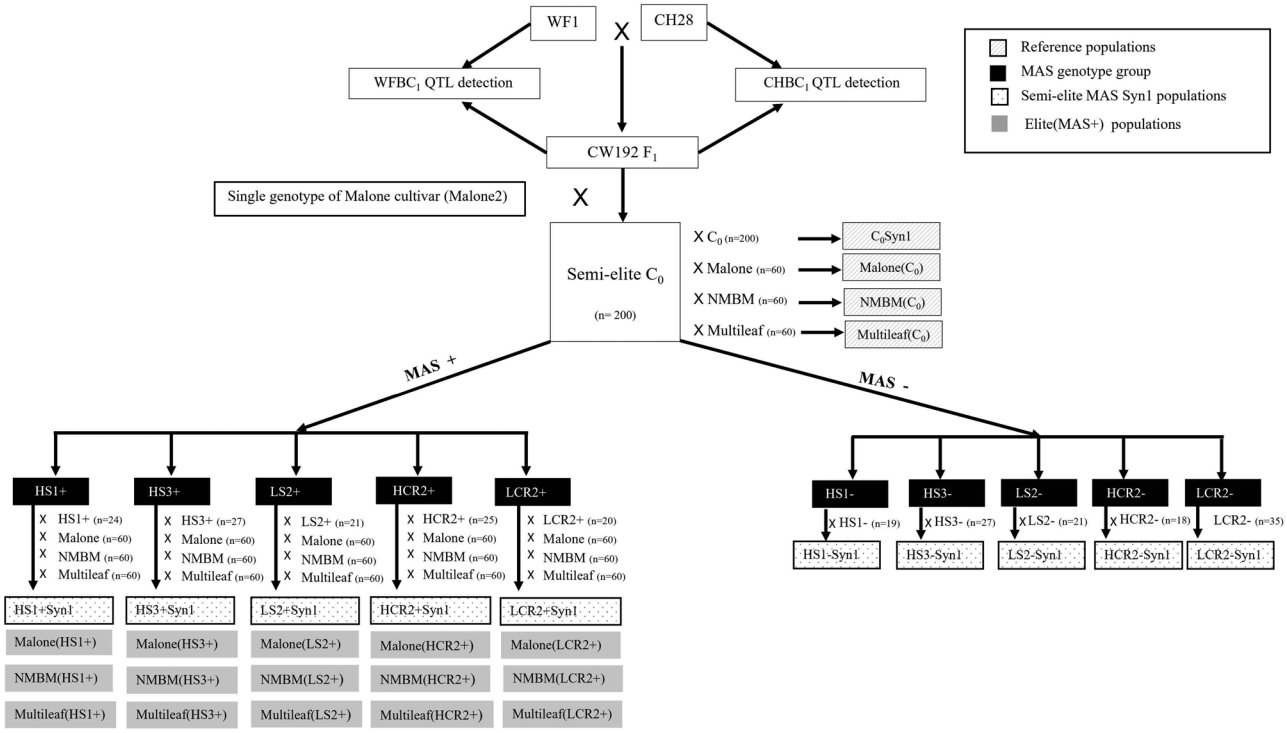

FIGURE 1 Scheme for development of populations used in the current study. Quantitative trait loci (QTL) detection was previously conducted in the Wisfal first-generation backcross ($WFBC_1$) and the Chilean first-generation backcross ($CHBC_1$) mapping populations, which were generated by mating two unimproved parent plants (WF1 and CH28) with one of their F_1 hybrid progeny (CW192). A single plant from the 'Malone' cultivar (Malone2) was then mated with CW192 (QTL donor parent) to generate a semi-elite C_0 population of 200 full-sib plants segregating for the QTL. Based on the C_0 genotyping results, QTL marker-assisted selection (MAS) was imposed. Ten MAS genotype groups of plants, each comprised of 18–35 individuals that possessed appropriate marker genotypes, were selected from the C_0 population and designated as HS1+ and HS1−, HS3+ and HS3−, LS2+ and LS2−, HCR2+ and HCR2−, and LCR2+ and LCR2− (black boxes). Each MAS genotype group designation indicated selection for (+) or against (−) one, two, or three marker alleles affiliated with high-shoot (HS), low-shoot (LS), high-crown-root (HCR), or low-crown-root (LCR) biomass. These MAS genotype groups of plants, and all 200 plants of the C_0 population, were randomly intercrossed by hand within their respective groups to produce 10 MAS Syn1 populations (dotted background boxes, e.g., HS1+Syn1) and a reference C_0Syn1 population (gray line background box). The MAS plants assigned to the HS1+, HS3+, LS2+, HCR2+, and LCR2+ MAS genotype groups, and all C_0 plants, were also crossed to 60 plants each from three elite alfalfa populations ('Malone', 'NuMex Bill Melton' [NMBM], and Multileaf) to generate 15 Elite(MAS+) populations (solid gray boxes; e.g., Malone(HS1+) and three affiliated unselected reference populations (gray line background boxes: Malone(C_0), NMBM(C_0), and Multileaf(C_0)

Melton (Ray et al., 2012; hereafter referred to as, NMBM) and an elite multifoliate and large-leaf germplasm (designated Multileaf) derived from the following cultivars: 'Amerileaf 721′ (America's Alfalfa), 'Dona Ana' (Melton, et al., 1985), and 'WL530' (W-L Alfalfas). The Malone, NMBM, and Multileaf populations were chosen based on the expectation that they would differ in resilience to drought stress. In this regard, the NMBM population was selected for high performance in both deficit-irrigated and well-watered environments (Ray et al., 2012). The Malone and Multileaf elite germplasms had not experienced any direct selection for productivity under drought-stress conditions. Furthermore, the Multileaf population was anticipated to be most sensitive to water stress given that it possessed greater leaf-to-stem ratio (i.e., greater transpirational water loss potential) than either NMBM or Malone. Fifteen populations produced from these crosses were designated as Malone(HS1+), NMBM(HS1+), Multileaf(HS1+), Malone(HS3+), NMBM(HS3+), etc., and will be collectively referred to as Elite(MAS+)(Figure 1; Table 2). Their three affiliated reference populations included Malone(C_0), NMBM(C_0), and Multileaf(C_0), which will be collectively referred to as Elite(C_0). No additional selection was practiced in the Elite(MAS+) or Elite(C_0) populations. The 60 plants representing the Malone, NMBM, and Multileaf elite populations were also randomly intermated to provide three elite check germplasms. A balanced composite of seed from each plant involved in each cross was used to represent each of the above 21 populations.

In total, 32 populations were developed for this study including 10 semi-elite MAS Syn1 populations and the semi-elite C_0Syn1 reference population (50% elite genetic background), 15 Elite(MAS+) populations and three Elite(C_0) ref-

TABLE 1 Simple sequence repeat (SSR) markers affiliated with 10 genome regions influencing alfalfa shoot and crown–root (CR) biomass during drought stress at Las Cruces, NM

SSR marker[a]	Donor parent	Biomass effect[b]	*M. sativa* linkage group[c]	Marker position on *M. sativa* composite map[c]	QTL position on *M. sativa* composite map[d]
		Mg ha^{-1}		cM	
AW86	CH28	Shoot (+0.104)	1C	54	70 (49–72)
AL22	WF1	Shoot (+0.068)	1A	72	72 (70–72)
BE137	WF1	Shoot (+0.045)	6A	57	51 (44–57)
BE140	WF1	Shoot (+0.057)	8A	7	7 (0–9)
BF228	WF1	Shoot (−0.094)	1B	5	5 (3–10)
AL29	WF1	Shoot (−0.094)	5B	30	30 (29–32)
AFca11	WF1	CR (+0.198)	6A	34	33 (32–34)
AW256	WF1	CR (+0.126)	7A	8	0 (0–19)
BG57	WF1	CR (−0.122)	3A	60	67 (60–69)
MTIC103	WF1	CR (−0.186)	8A	13	18 (10–23)

[a]Primer pair sequences for each marker are provided in Supplemental Table S1.
[b]Shoot biomass effect (in parentheses) determined at the specified marker locus based on six drought-stressed environments at Las Cruces, NM (Ray et al., 2015). Crown-root biomass effect (in parenthesis) determined at the specified marker locus based on a single destructive harvest in 2007 when the study reported by Ray et al. (2015) was terminated (results provided in Supplemental Tables S2, S3, and S4 of this report).
[c]Linkage group and position of the targeted marker based on an *M. sativa* composite genetic map (Ray et al., 2015), where linkage groups A or B were inherited from the WF1 parent, while groups C and D were inherited from the CH28 parent.
[d]Position of a biomass quantitative trait loci (QTL) on an *M. sativa* composite genetic map (Ray et al., 2015) and its confidence interval (in parentheses) as defined by values within one unit of the maximum logarithm of the odds.

erence populations (75% elite genetic background), and three elite checks. A summary of all populations evaluated in this study is provided in Table 2 and a schematic diagram illustrating their development is provided in Figure 1.

2.4 | Field experimental design and irrigation management

For field-based phenotyping, the 32 populations were planted at the New Mexico State University Leyendecker Plant Science Research Center, near Las Cruces, NM, USA. This site was known to possess heterogeneity in soil texture and, hence, water-holding capacity, which could result in spatial variation for alfalfa forage yield particularly under water-stressed conditions. To maximize precision of the alfalfa forage yield data to be collected at this site, soil water-holding capacity heterogeneity was identified by planting a wheat (*Triticum aestivum* L.) cover crop in spring 2010 before establishing the alfalfa research plots. Irrigation was terminated after the wheat was well established, and severity of wilting and drought-induced senescence were visually scored and mapped following the approach described by Ray et al. (2015). The wheat cover crop was subsequently mowed and incorporated into the soil. In late September 2010, fertilizer was applied to this field at a rate of 336 kg ha^{-1} 11–52–0 (N–P–K). In mid-October 2010, eight replicates of the 32 alfalfa populations were planted according to a randomized complete block design within a region of the field that demonstrated relatively uniform soil water-holding capacity based on the previous wheat drought-stress phenotyping. All alfalfa populations were seeded with a mechanical drill in three-row plots, 1.5 m long, at a rate of 400 seeds per plot. Rows within plots were spaced 30 cm apart and plots were spaced 60 cm apart. The field trial was planted on a Glendale clay loam (Fine-silty, mixed, superactive, calcareous, thermic Typic Torrifluvents) (pH = 8.0) and received standard irrigation management (i.e., application of ~70 mm of water at 14-d intervals) for stand establishment during fall 2010. Deficit flood irrigation management for the duration of the 3-yr study was implemented starting in spring 2011 (i.e., 50% of standard irrigation rates where 70 mm of water was applied approximately every 28 d throughout the growing season). The herbicides Clethodim 2E and Poast Plus were applied at a rate of 700 g ha^{-1} and 2.63 L ha^{-1}, respectively, in spring of 2011 to control volunteer wheat. In June of each year, Trifluralin HF was applied at a rate of 2.8 L ha^{-1} to control summer annual grasses.

2.5 | Forage yield phenotyping

Forage biomass was harvested from the 32 populations six times each year (late April through October) during 2011–2013. During each alfalfa regrowth cycle, visible symptoms of water stress (i.e., wilting) were allowed to progress until drought-induced leaf senescence was observed in the lower

TABLE 2 Description of 32 alfalfa populations evaluated in this study

Population	Parentage
C_0Syn1	Intermated 200 plants of the Cycle 0 base population. Genetic background: 50% 'Malone', 25% *M. sativa* subsp. *sativa* var. Chilean, and 25% *M. sativa* subsp. *falcata* 'WISFAL'
HS1+Syn1	Intermated 24 C_0 plants of HS1+ MAS genotype group possessing high shoot biomass marker allele, AW86
HS1−Syn1	Intermated 19 C_0 plants of HS1− MAS genotype group lacking high shoot biomass marker allele, AW86
HS3+Syn1	Intermated 27 C_0 plants of HS3+ MAS genotype group possessing high shoot biomass marker alleles, AL22, BE137, BE140
HS3−Syn1	Intermated 27 C_0 plants of HS3− MAS genotype group lacking high shoot biomass marker alleles, AL22, BE137, BE140
LS2+Syn1	Intermated 21 C_0 plants of LS2+ MAS genotype group possessing low shoot biomass marker alleles, BF228, AL29
LS2−Syn1	Intermated 21 C_0 plants of LS2− MAS genotype group lacking low shoot biomass marker alleles, BF228, AL29
HCR2+Syn1	Intermated 25 C_0 plants of HCR2+ MAS genotype group possessing high crown–root biomass marker alleles, AFca11, AW256
HCR2−Syn1	Intermated 18 C_0 plants of HCR2− MAS genotype group lacking high crown–root biomass marker alleles, AFca11, AW256
LCR2+Syn1	Intermated 20 C_0 plants of LCR2+ MAS genotype group possessing low crown–root biomass marker alleles, BG57, MTIC103
LCR2−Syn1	Intermated 35 C_0 plants of LCR2− MAS genotype group lacking low crown–root biomass marker alleles, BG57, MTIC103
'Malone'	Malone population regenerated by intermating 60 individuals from Malone cultivar
Malone(C_0)	60 Malone plants mated with all C_0 plants; reciprocal mating, balanced seed composite
Malone(HS1+)	60 Malone plants mated with HS1+ MAS genotype group; reciprocal mating, balanced seed composite
Malone(HS3+)	60 Malone plants mated with HS3+ MAS genotype group; reciprocal mating, balanced seed composite
Malone(LS2+)	60 Malone plants mated with LS2+ MAS genotype group; reciprocal mating, balanced seed composite
Malone(HCR2+)	60 Malone plants mated with HCR2+ MAS genotype group; reciprocal mating, balanced seed composite
Malone(LCR2+)	60 Malone plants mated with LCR2+ MAS genotype group; reciprocal mating, balanced seed composite
'NuMex Bill Melton' (NMBM)	NMBM population regenerated by intermating 60 individuals from NMBM cultivar
NMBM(C_0)	60 NMBM plants mated with all C_0 plants; reciprocal mating, balanced seed composite
NMBM(HS1+)	60 NMBM plants mated with HS1+ MAS genotype group; reciprocal mating, balanced seed composite
NMBM(HS3+)	60 NMBM plants mated with HS3+ MAS genotype group; reciprocal mating, balanced seed composite
NMBM(LS2+)	60 NMBM plants mated with LS2+ MAS genotype group; reciprocal mating, balanced seed composite
NMBM(HCR2+)	60 NMBM plants mated with HCR2+ MAS genotype group; reciprocal mating, balanced seed composite
NMBM(LCR2+)	60 NMBM plants mated with LCR2+ MAS genotype group; reciprocal mating, balanced seed composite
Multileaf	Multileaf population regenerated by intermating 60 individuals from that elite germplasm
Multileaf(C_0)	60 Multileaf plants mated with all C_0 plants; reciprocal mating, balanced seed composite
Multileaf(HS1+)	60 Multileaf plants mated with HS1+ MAS genotype group; reciprocal mating, balanced seed composite
Multileaf(HS3+)	60 Multileaf plants mated with HS3+ MAS genotype group; reciprocal mating, balanced seed composite
Multileaf(LS2+)	60 Multileaf plants mated with LS2+ MAS genotype group; reciprocal mating, balanced seed composite
Multileaf(HCR2+)	60 Multileaf plants mated with HCR2+ MAS genotype group; reciprocal mating, balanced seed composite
Multileaf(LCR2+)	60 Multileaf plants mated with LCR2+ MAS genotype group; reciprocal mating, balanced seed composite

canopy for a vast majority of the field plots and the wilted shoots were unable to recover full turgidity overnight. At that time, severity of water stress was generally characterized by measuring leaf relative water content (LRWC; Turner, 1981), where 15 fully expanded leaves were collected near the shoot apex at solar noon from each of the C_0Syn1, Malone, Multileaf, and NMBM reference populations. Leaf tissues were collected from individual plots present in four random replicates of the study. Forage yield data collection commenced within 1 or 2 d thereafter. Based on observed drought response in the plants, total shoot biomass was collected from all experimental plots to a 5-cm stubble height every 24–35 d using a research plot flail harvester with an automated weighing system. During each harvest, fresh forage samples were regularly

collected from multiple plots, dried at 50 ˚C for at least 48 h, and used to adjust yield data to a dry matter basis. Seasonal forage biomass production was determined by summing the yield data over all harvests within each year following a similar approach to that described by Bhandari et al. (2007) and Madril et al. (2008).

2.6 | Data analysis

Seasonal forage yield data (Mg ha^{-1}) adjusted to a dry matter basis were subjected to statistical analysis using the general linear model procedure (PROC GLM) and the mixed linear model procedure (PROC MIXED) of SAS v9.3 (SAS Institute). Data were analyzed as a balanced randomized complete block design in individual years as well as a split plot in time over years where populations were assigned as the whole plot and years as the split plot (Nyquist & Baker, 1991). Blocks were considered a random effect, whereas populations and years were fixed effects. Evaluation of fit statistics for multiple mixed models possessing different variance–covariance structures for yield data over years suggested that a PROC MIXED repeated measures analysis using a first-order antedependence [ANTE(1)] covariance structure best fit the alfalfa biomass data. For comparative purposes, analyses of the alfalfa data over years were conducted using two model procedures including the previously mentioned PROC MIXED repeated measures analysis with ANTE(1) covariance structure and a traditional PROC GLM mixed-model ANOVA approach, which assumed compound symmetry (CS) covariance structure. To evaluate the effect of the biomass marker alleles on forage yield in differing elite alfalfa genetic backgrounds, an α level of 0.1 was established to declare significance. This relaxed threshold was deemed reasonable because, in many cases, only 50% of the members of any given population were expected to possess a given marker allele.

3 | RESULTS AND DISCUSSION

3.1 | Crown-root biomass results from prior work in the CHBC$_1$ population

In a previous study conducted during 2005–2007, multiple QTL associated with alfalfa forage biomass were identified under deficit irrigation management (Ray et al., 2015). When that study was terminated in 2007, CR biomass data were also obtained from the CHBC$_1$ population. Those results have not been reported and will be described here. Significant variation ($P < .0001$) for CR biomass was detected among the half-sib families derived from the CH28 and WF1 parents, their CW192F$_1$ hybrid, and 96 CHBC$_1$ plants (Supplemental Tables S2 and S3). Interval mapping detected four QTL in the CW192 plant, of which two increased and two decreased CR biomass (Supplemental Table S4). Two additional QTL were identified in the CH28 recurrent parent, both of which reduced CR biomass. Given that CR biomass and shoot biomass demonstrated a significant positive genetic correlation in CHBC$_1$ ($r = 0.71$; $p < .01$), QTL affiliated with both traits were evaluated for their potential to affect forage productivity during drought stress in more elite genetic backgrounds.

3.2 | Seasonal dry matter forage yield under deficit irrigation management

Growing season environmental conditions (1 March to 31 October) during this 3-yr study were as follows. Average daily maximum and minimum temperatures ranged from 30 to 31 ˚C and 11 to 12 ˚C, respectively. Total precipitation was 9.5, 12.3, and 13.7 cm in 2011, 2012, and 2013, respectively. The LRWC of the deficit-irrigated C$_0$Syn1, Malone, Multileaf, and NMBM reference populations averaged 72, 62, and 60% over all harvests in 2011, 2012, and 2013, respectively. In a neighboring well-watered irrigation treatment study that was planted at the same time as the deficit-irrigated study, these same populations demonstrated higher average LRWCs of 85% in 2011 and 84% in both 2012 and 2013. Compared with the well-watered study, the deficit-irrigated plots also exhibited yield reductions of 40, 41, and 31% in 2011, 2012, and 2013, respectively. Collectively, the notable reductions in LRWC and forage yield indicated that the deficit-irrigated study plots experienced considerable water stress over the duration of this study. Similar deficit irrigation management approaches in alfalfa are known to cause severe water stress as demonstrated by substantial reductions in yield, shoot height, LRWC, photosynthesis, stomatal conductance, and higher canopy temperatures (Ray et al., 1999a, 1999b; Maruthavanan et al., 2007, 2009).

Seasonal forage biomass of 32 alfalfa populations varied significantly within and across years under continuous deficit irrigation management based on the PROC MIXED analysis ($P < .0001$) and the PROC GLM analysis (Table 3). A significant genotype × year interaction was also detected in both analyses ($P = .02$ and .04 for the MIXED and GLM analyses, respectively). Based on the PROC GLM analysis Type 1 sums of squares, this interaction was eight times smaller than the genotype main effect, therefore, our discussion will focus largely on results of data analyses over years.

Biomass data were balanced within and across years. Hence, means provided by the PROC GLM analysis and least-square means based on the PROC MIXED analysis were equivalent. Mean seasonal dry matter yield of all populations within each year and across years is presented in Table 4. Three-year average yields ranged from 7.82 to 14.00 Mg ha^{-1}. Overall, the semi-elite C$_0$Syn1 reference population and MAS Syn1 populations (50% elite Malone genetic background) pos-

TABLE 3 Mean squares based on a general linear model mixed-model analysis for seasonal dry matter yield (Mg ha^{-1}) of 32 alfalfa populations evaluated under continuous deficit irrigation management in 2011, 2012, and 2013 at Las Cruces, NM

Source	df	2011	2012	2013	df	Across years
		Mg ha^{-1}				Mg ha^{-1}
Block	7	41.3†	83.4†	34.5†	7	132.9†
Genotype	31	21.5†	34.8†	28.9†	31	75.7†
Genotype × block	217	5.2	13.9	4.6	217	17.4†
Year	–	–	–	–	2	319.1†
Genotype × year	–	–	–	–	62	4.7*
Residual	–	–	–	–	448	3.5

*Significant at the .05 probability level. †Significant at the .0001 probability level

sessed the lowest yields. Progressively higher yields were noted for the 15 Elite(MAS+) populations, which possessed a 75% elite genetic background resulting from mating the five semi-elite MAS+ genotype groups with the three elite germplasms. Over the 3 yr of this study, the NMBM population demonstrated the highest average yield, while the Malone and Multileaf elite checks ranked 10 and 11, respectively.

3.3 | MAS Syn1 and C_0Syn1 populations

The Malone cultivar was specifically chosen as the elite parent for developing the C_0 population because (a) it had not experienced any direct selection for productivity under water-deficit conditions, and (b) 77% of its parentage traced to Chilean genetic sources (Melton et al., 1989a). Importantly, nine of the 10 biomass marker alleles evaluated in this study were detected in the $CHBC_1$ population, which possessed 75% Chilean genetic background. Hence, we hypothesized that introgression of these markers into a potentially drought-sensitive elite population with a genetic background similar to $CHBC_1$ might facilitate detection and characterization of QTL marker phenotypic effects. In this regard, we noted that a majority (i.e., eight) of the MAS Syn1 populations (50% elite Malone background), including three that were anticipated to possess reduced biomass, exhibited forage yields that were numerically greater than the C_0Syn1 reference population (Table 4). Superior performance for five of these populations (i.e., HS1+Syn1, HS3+Syn1, HCR2+Syn1, LS2−Syn1, and LCR2−Syn1) could potentially represent the effect of MAS for favorable alleles or against unfavorable alleles. Given that C_0Syn1 was derived by intermating 200 C_0 plants, such outcomes could also reflect random variation attributed to small sample sizes of selected C_0 plants ($n = 18$–35) that possessed appropriate marker genotypes and that were intermated to generate the 10 MAS Syn1 populations. Nevertheless, results of PROC GLM and PROC MIXED analyses involving only the MAS Syn1 and C_0Syn1 populations over 3 yr both indicated that the LCR2−Syn1 and HS3+Syn1 MAS populations possessed significantly ($P < .1$) greater yield than C_0Syn1 (data not shown).

Implementation of QTL MAS largely resulted in expected phenotypic responses within and over years for the MAS Syn1 populations (Table 5). In all 3 yr of the study, MAS Syn1 populations selected for HS or HCR biomass markers performed better than their respective populations derived by selecting against those markers. Similarly, a population derived by selecting against LCR biomass markers consistently outperformed a population derived by selecting for those markers. Little response was observed for LS biomass marker selection. Based on the PROC GLM mixed-model CS covariance structure analysis over 3 yr, MAS for or against HS3, HCR2, and LCR2 markers significantly ($P < .1$) affected forage biomass, while selection directed at the HS1 marker approached significance ($P = .11$). Based on the PROC MIXED repeated measures ANTE1 covariance structure analysis over 3 yr, MAS for or against HS3 markers significantly ($P < .1$) affected forage biomass, while MAS directed at the HCR2 and LCR2 markers approached significance ($P \leq .12$). Selection response over 3 yr varied from a significant 19.44% difference between the HS3+Syn1 and HS3−Syn1 populations to a nonsignificant −0.92% difference between the LS2+Syn1 and LS2−Syn1 populations.

3.4 | Elite(MAS+) and Elite(C_0) populations

To test the consistency of QTL marker allele effects, the semi-elite MAS plants assigned to the MAS+ genotype groups (HS1+, HS3+, LS2+, HCR2+, and LCR2+) and the C_0 population were each crossed to the elite Malone, NMBM, and Multileaf germplasms (Table 2; Figure 1). These matings generated 15 Elite(MAS+) populations consisting of five Malone(MAS+), five NMBM(MAS+), five Multileaf(MAS+) populations and three Elite(C_0) reference populations including Malone(C_0), NMBM(C_0), and Multileaf(C_0). As previously indicated, the elite Malone germplasm was derived primarily from Chilean genetic sources. The NMBM germplasm was derived primarily from Turkistan genetic sources (Melton et al., 1989b; Ray et al., 2012), and Multileaf possessed a broad genetic base

TABLE 4 Mean seasonal dry matter yields (Mg ha^{-1}) of 32 alfalfa populations evaluated under deficit irrigation management over 3 yr at Las Cruces, NM

Population	Yield			
	2011	2012	2013	3-yr avg.
	Mg ha^{-1}			
'NuMex Bill Melton' (NMBM)	15.03* (1)[a]	14.10* (6)	12.86* (1)	14.00* (1)
Multileaf(LS2+)	13.36* (10)	15.42* (1)	12.54* (2)	13.77* (2)
NMBM(HS1+)	14.78* (2)	13.72* (8)	12.48* (3)	13.66* (3)
NMBM(HS3+)	13.80* (7)	14.88* (2)	12.02* (8)	13.57* (4)
NMBM(HCR2+)	13.40* (9)	14.51* (4)	12.07* (4)	13.33* (5)
Malone(HS3+)	13.91* (5)	14.53* (3)	11.18* (11)	13.21* (6)
NMBM(C0)	13.87* (6)	13.25* (9)	11.55* (10)	12.89* (7)
Multileaf(HS1+)	14.18* (3)	12.38* (14)	12.03* (7)	12.87* (8)
Multileaf(LCR2+)	12.58 (14)	13.96* (7)	12.04* (6)	12.86* (9)
Malone	13.59* (8)	13.15* (10)	11.75* (9)	12.83* (10)
Multileaf	11.51 (20)	12.61* (13)	12.07* (5)	12.06* (11)
Malone(LS2+)	11.46 (21)	14.17* (5)	10.53 (14)	12.05* (12)
Multileaf(HCR2+)	12.62 (13)	12.73* (12)	10.56 (13)	11.97* (13)
Multileaf(HS3+)	12.64 (12)	11.85 (15)	10.08 (17)	11.52 (14)
NMBM(LCR2+)	12.84 (11)	11.25 (20)	10.31 (15)	11.47 (15)
Multileaf(C0)	11.74 (17)	11.81 (16)	10.84 (12)	11.46 (16)
NMBM(LS2+)	13.96* (4)	10.38 (23)	9.98 (19)	11.44 (17)
Malone(LCR2+)	11.39 (22)	12.80* (11)	9.91 (21)	11.37 (18)
Malone(HS1+)	11.65 (18)	10.59 (22)	10.30 (16)	10.85 (19)
Malone(HCR2+)	11.13 (24)	11.31 (19)	9.96 (20)	10.80 (20)
LCR2-Syn1	11.99 (15)	11.53 (17)	8.41 (22)	10.64 (21)
HS3+Syn1	11.89 (16)	11.46 (18)	8.21 (24)	10.52 (22)
Malone(C0)	10.52 (25)	10.74 (21)	10.03 (18)	10.43 (23)
LS2-Syn1	11.62 (19)	10.38 (24)	8.03 (25)	10.01 (24)
LS2+Syn1	11.15 (23)	10.29 (25)	8.31 (23)	9.92 (25)
HCR2+Syn1	10.35 (28)	10.24 (26)	7.70 (28)	9.43 (26)
LCR2+Syn1	10.51 (26)	9.38 (27)	7.74 (27)	9.21 (27)
HS1+Syn1	10.39 (27)	9.10 (29)	7.78 (26)	9.09 (28)
HS3-Syn1	9.67 (30)	9.29 (28)	7.46 (29)	8.81 (29)
C0Syn1	10.02 (29)	8.91 (30)	7.36 (30)	8.76 (30)
HCR2-Syn1	9.02 (32)	8.18 (31)	6.64 (32)	7.95 (31)
HS1-Syn1	9.06 (31)	7.49 (32)	6.91 (31)	7.82 (32)
Mean	12.05	11.76	9.99	11.27
LSD0.1	1.88	3.07	1.78	1.98
CV (%)	18.94	31.7	21.57	16.59

*Not significantly different from largest value in each column based on analysis of 32 populations and LSD = 0.1.
[a]Forage yield rank (in parenthesis) within each column. Data sorted by 3-yr avg. yield.

from Indian, African, Chilean, and Turkistan genetic sources (unpublished data, 2007). Crosses with the unselected C_0 population led to yield reduction in all three elite genetic backgrounds (Figure 2). Notably, Malone(C_0) and Multileaf(C_0) yielded less than their respective Malone(MAS+) and Multileaf(MAS+) populations. Among the Elite(MAS+) populations, we also observed that the effect of biomass markers on forage yield varied across the genetic backgrounds as discussed below.

TABLE 5 Pairwise contrast estimates between alfalfa Syn1 populations derived from divergent DNA marker-assisted selection (MAS), their affiliated *P* values (in parentheses), and percentage selection response based on seasonal dry matter yield (Mg ha^{-1}) over 3 yr under deficit irrigation management at Las Cruces, NM

Contrast between divergent MAS Syn1 populations[a]	Difference between means[b]				Selection response			
	2011	2012	2013	Over 3 yr	2011	2012	2013	Over 3 yr
	Mg ha^{-1}				%			
HS1+Syn1 vs. HS1−Syn1	1.33 (VC, .13)	1.60 (VC, .19)	0.86 (VC, .22)	1.26 (CS, .11) (ANTE1, .17)	14.63	21.47	12.56	16.21
HS3+Syn1 vs. HS3−Syn1	**2.22 (VC, .01)**	**2.16 (VC, .08)**	0.74 (VC, .29)	**1.71 (CS, .03) (ANTE1, .06)**	22.97	23.30	10.02	19.44
LS2+Syn1 vs. LS2−Syn1	−0.46 (VC, .59)	−0.08 (VC, .94)	0.27 (VC, .69)	−0.09 (CS, .90) (ANTE1, .92)	−4.01	−0.82	3.43	−0.92
HCR2+Syn1 vs. HCR2−Syn1	1.32 (VC, .13)	2.05 (VC, .10)	1.06 (VC, .13)	**1.48 (CS, .06)** (ANTE1, .11)	14.74	25.06	16.05	18.65
LCR2+Syn1 vs. LCR2−Syn1	**−1.47 (VC, .09)**	**−2.14 (VC, .08)**	−0.67 (VC, .34)	**−1.43 (CS, .07)** (ANTE1, .12)	−12.32	−18.58	−8.00	−13.45

Note. Bold font indicates a < .1 significance level met.

[a]Pairwise contrast between alfalfa Syn1 populations derived from divergent MAS for (+) or against (−) DNA markers affiliated with high shoot (HS), low shoot (LS), high crown–root (HCR), or low crown–root (LCR) biomass production. Refer to Table 2 for detailed information affiliated with MAS Syn1 populations.

[b]*P* values affiliated with individual year analyses were based on a mixed model and standard variance components (VC). *P* values affiliated with the over-years analyses were based on two procedures including (a) a traditional PROC GLM mixed model ANOVA approach with compound symmetry (CS) covariance structure and (b) a PROC MIXED repeated measures analysis with first-order antedependence (ANTE1) covariance structure. Fit statistics of multiple mixed models possessing different variance–covariance structures suggested that the ANTE1 covariance structure best fit the alfalfa biomass data.

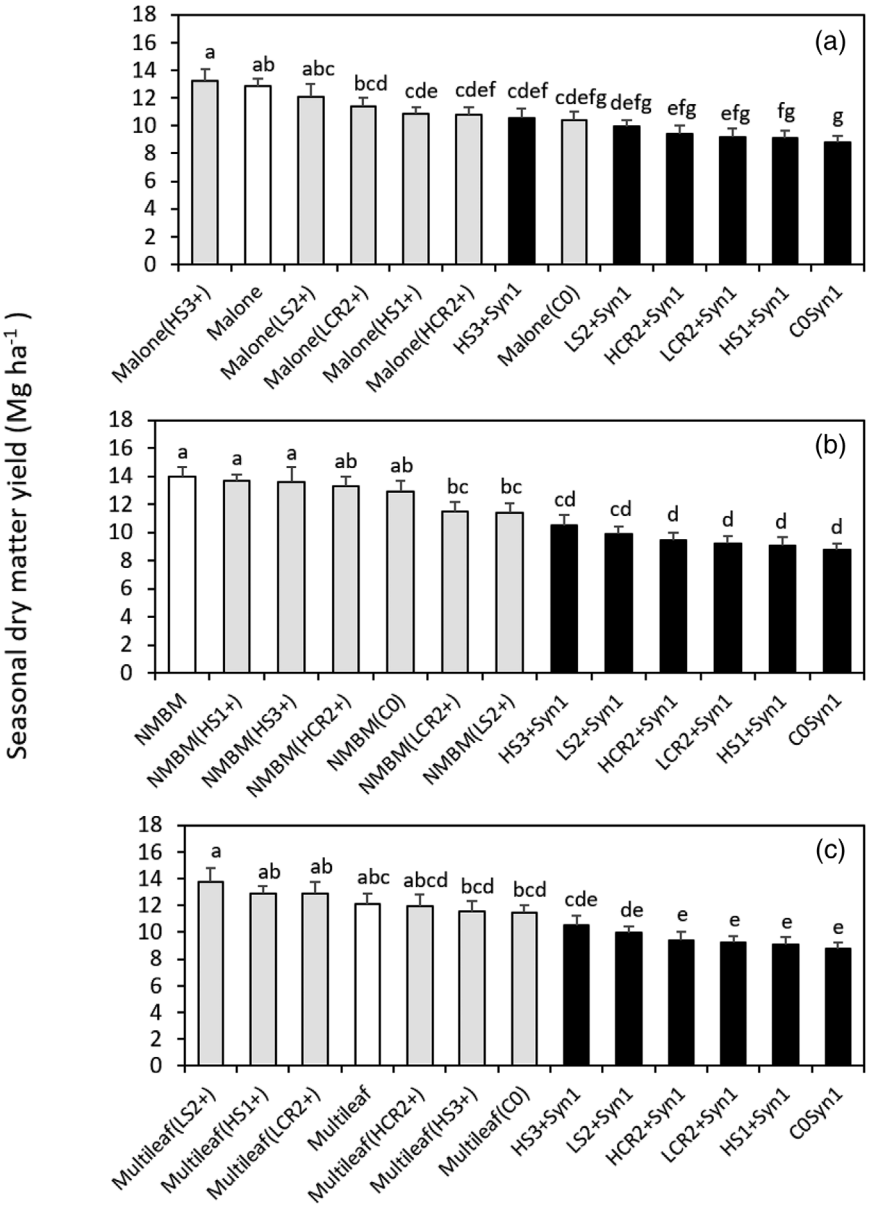

FIGURE 2 Mean seasonal dry matter yield over 3 yr (± SE) of the C_0Syn1 and marker-assisted selection Syn1 (MAS+Syn1) populations (black bar), Elite(C_0) and Elite(MAS+) populations (gray bar), and three elite parent populations (white bar). Semi-elite C_0Syn1 and MAS+Syn1 populations possessed a 50% elite 'Malone' genetic background. Elite(C_0) and Elite(MAS+) populations possessed 75% elite genetic background and were generated by mating all semi-elite plants of the C_0 base population and all semi-elite plants assigned to the HS1+, HS3+, LS2+, HCR2+, and LCR2+ MAS genotype groups with three elite populations ('Malone', Panel A; 'NuMex Bill Melton' [NMBM], Panel B; and Multileaf, Panel C). Means with same letter in each panel do not differ significantly at $\alpha = 0.1$

3.5 | MAS+ and C_0 populations in the Malone background

When the C_0 population and five MAS+ genotype groups of plants were again mated to the Malone cultivar, forage yields of these populations (75% Malone genetic background) increased ~2 Mg ha^{-1} on-average over 3 yr (Figure 2a; Table 4). Notably, the five Malone(MAS+) and Malone(C_0) populations and their corresponding MAS+ Syn1 and C_0Syn1 populations ranked similarly for forage yield (Spearman rank correlation (r_s = 0.83; $P < .05$), indicating a relatively consistent phenotypic effect of the biomass markers in the Malone background. Malone(HS3+) performed significantly better than Malone(LCR2+), Malone(HS1+), and Malone(HCR2+) with a maximum yield superiority of 26% over the Malone(C_0) unselected reference population. Malone(HS3+) also demonstrated a nonsignificant 3% yield advantage over the Malone cultivar.

3.6 | MAS+ and C_0 populations in the NMBM background

When the C_0 and five MAS+ genotype groups of plants were mated to the NMBM cultivar, forage yields increased ~3 Mg ha^{-1} on-average over 3 yr (Figure 2b, Table 4). These five NMBM(MAS+) populations and NMBM(C_0) possessed a 50% NMBM and 25% Malone genetic background and showed no similarity for forage yield rankings (r_s = −0.08; $P > .8$) with their corresponding MAS+ and C_0 Syn1 populations, which possessed a 50% Malone genetic background. The effects of the biomass markers within the drought-resilient NMBM genetic background were in greatest

agreement with expectations based on the report of Ray et al. (2015), where marker effects were initially characterized. In the NMBM background, populations with HS and HCR biomass markers performed superior to the NMBM(C_0) population and populations containing LS or LCR biomass markers. These differences were significant between the higher yielding NMBM(HS1+) and NMBM(HS3+) populations when compared with the lower yielding NMBM(LCR2+) and NMBM(LS2+) populations, with a 19% yield difference observed between NMBM(HS1+) and NMBM(LS2+). Three NMBM(MAS+) populations and NMBM(C_0) performed similar to but did not exceed the yield of the NMBM cultivar.

3.7 | MAS+ and C_0 populations in the Multileaf background

Forage yields of the Multileaf(C_0) and five Multileaf(MAS+) populations yielded, on average, ~3 Mg ha^{-1} more than their corresponding C_0Syn1 and MAS+ Syn1 populations (Figure 2c, Table 4). No similarities were detected for ranking of forage yield among the C_0Syn1 and MAS+ Syn1 populations and the Multileaf-derived populations (r_s = 0.20; $P > .7$). All Multileaf(MAS+) and Multileaf(C_0) populations yielded similar to the elite Multileaf germplasm, with three populations numerically exceeding the yield of Multileaf by 6–14%. The highest yielding population was Multileaf(LS2+), which significantly outperformed Multileaf(HS3+) and Multileaf(C_0) by 19 and 20%, respectively. Ironically, two of the lowest yielding populations possessed the HCR2+ and HS3+ markers. The observed outcomes of the Multileaf-derived populations suggest that genetic background can affect the relative phenotypic effect of marker alleles. This is not unexpected given the distinctly different shoot canopy architecture and genetic background affiliated with the Multileaf population.

4 | CONCLUSIONS

To date, QTL MAS for biomass productivity during drought has not been reported in alfalfa. Here we describe the first validation study of QTL MAS in alfalfa for drought resilience. Two key goals of this study were to (a) validate the effect of several alfalfa biomass QTL that were originally detected in unimproved germplasm and (b) determine their potential to improve forage productivity of diverse elite germplasm during drought. Toward this end, application of DNA MAS improved forage biomass productivity of some semi-elite and elite alfalfa germplasms under deficit irrigation management. In semi-elite MAS Syn1 populations possessing a 50% elite Malone genetic background, MAS for one to three DNA markers associated with HS or HCR biomass resulted in populations that outperformed those that lacked the favorable biomass markers. Furthermore, selection against markers associated with low CR biomass production tended to improve forage productivity relative to populations that possessed those markers. The biomass differences mentioned above were not always significant, which likely reflect the polygenic nature of forage yield. For instance, each of the 10 QTL evaluated in the current study accounted for 3–8% of the variation in forage or CR biomass in the $CHBC_1$ population (Ray et al., 2015; Supplemental Table S4). These outcomes also demonstrate challenges that may be encountered when attempting to measure the effect of biomass QTL in drought-stressed field experiments. Despite our use of eight replications, reduced precision of yield data was apparent in the study, where CVs ranged from 19 to 32% (Table 4). Such outcomes are commonly observed in deficit-irrigated alfalfa yield trials, where CVs are about twice as large as those typically associated with well-watered trials (Ray et al., 1999a, 1999b, 2015). The higher CVs observed in our study can be partially attributed to the presence of heterogeneous soils within experimental blocks and the magnified effect of spatial variation in soil texture and soil water-holding capacity on forage yield under water-deficit field conditions.

The biomass markers were also evaluated for phenotypic effects in three different elite genetic backgrounds, where semi-elite MAS+ plants assigned to the HS1+, HS3+, LS2+, HCR2+, and LCR2+ MAS genotype groups and the C_0 population were each crossed to the elite Malone, NMBM, and Multileaf germplasms. Among these Elite(MAS+) populations that possessed 75% elite germplasm, generally higher yields (likely resulting from heterosis) were observed in the NMBM–Malone and Multileaf–Malone hybrid backgrounds relative to the Malone–Malone backcross population. In the Malone and Multileaf backgrounds, which had not previously experienced direct selection pressure for forage productivity during drought, some Malone(MAS+) and Multileaf(MAS+) populations possessed numerically higher yields than their elite parent. In the drought-resilient elite NMBM genetic background, four NMBM-derived populations performed similar to but did not exceed the yield of the NMBM cultivar. However, performance of NMBM(MAS+) populations was notable in that they closely agreed with expectations of marker effects based on the report of Ray et al. (2015). That is, NMBM-derived populations with HS and HCR biomass markers performed superior to the reference NMBM(C_0) population and populations containing LS or LCR biomass markers. In all three elite backgrounds, significant differences in forage yield (ranging from 19 to 26%) were detected among some Elite(MAS+) and Elite(C_0) populations. However, the effect of biomass markers on forage yield varied across the three genetic backgrounds. Collectively, the outcomes of this study indicate that MAS can significantly affect forage biomass of elite alfalfa germplasm in drought-stressed environments. However, if biomass QTL are detected in donor

germplasm that is genetically dissimilar to targeted elite populations, characterization of both favorable and unfavorable donor alleles may be warranted within elite backgrounds of interest to confirm their phenotypic effects. Subsequently, MAS within the targeted elite germplasm for favorable donor alleles (incremental increase) and against unfavorable alleles (rapid purging) should optimize opportunities to improve alfalfa forage productivity in drought-stressed environments.

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AUTHOR CONTRIBUTIONS

Lovepreet Singh: Data curation; Formal analysis; Writing – original draft; Writing – review & editing. Chris Pierce: Conceptualization; Data curation; Methodology; Project administration. Nicholas Santantonio: Data curation; Formal analysis; Methodology; Writing – review & editing. Robert Steiner: Formal analysis; Writing – review & editing. Don Miller: Conceptualization; Funding acquisition; Resources; Writing – review & editing. Jon Reich: Funding acquisition; Project administration; Resources; Writing – review & editing. Ian M. Ray: Conceptualization; Data curation; Formal analysis; Funding acquisition; Methodology; Project administration; Resources; Supervision; Validation; Writing – original draft.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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REFERENCES

Annicchiarico, P., Nazzicari, N., Li, X., Wei, Y., Pecetti, L., & Brummer, E. C. (2015). Accuracy of genomic selection for alfalfa biomass yield in different reference populations. *BMC*, *16*, 1020. https://doi.org/10.1186/s12864-015-2212-y

Bhandari, H. S., Pierce, C. A., Murray, L. W., & Ray, I. M. (2007). Combining abilities and heterosis for forage yield among high-yielding accessions of the alfalfa core collection. *Crop Science*, *47*, 665–673. https://doi.org/10.2135/cropsci2006.06.0398

Bingham, E. T. (1993). Registration of WISFAL alfalfa (*Medicago sativa* subsp. *falcata*) tetraploid germplasm derived from diploids. *Crop Science*, *33*, 217–218. https://doi.org/10.2135/cropsci1993.0011183X003300010064x

Blum, A. (2005). Drought resistance, water-use efficiency, and yield potential—Are they compatible, dissonant, or mutually exclusive? *Australian Journal of Agricultural Research*, *56*, 1159–1168. https://doi.org/10.1071/AR05069

Blum, A. (2009). Effective use of water (EUW) and not water-use efficiency (WUE) is the target of crop yield improvement under drought stress. *Field Crops Research*, *112*, 119–123. https://doi.org/10.1016/j.fcr.2009.03.009

Cattivelli, L., Rizza, F., Badeck, F.-W., Mazzucotelli, E., Mastrangelo, A. M., Francia, E., Marè, C., Tondelli, A., & Stanca, A. M. (2008). Drought tolerance improvement in crop plants: An integrated view from breeding to genomics. *Field Crops Research*, *105*, 1–14. https://doi.org/10.1016/j.fcr.2007.07.004

FAO. (2021). Drought. https://www.fao.org/land-water/water/drought/en/

Gudys, K., Guzy-Wrobelska, J., Janiak, A., Dziurka, M. A., Ostrowska, A., Hura, K., Jurczyk, B., Żmuda, K., Grzybkowska, D., Śróbka, J., Urban, W., Biesaga-Koscielniak, J., Filek, M., Koscielniak, J., Mikołajczak, K., Ogrodowicz, P., Krystkowiak, K., Kuczyńska, A., Krajewski, P., & Szarejko, I. (2018). Prioritization of candidate genes in QTL regions for physiological and biochemical traits underlying drought response in barley (*Hordeum vulgare* L.). *Frontiers in Plant Science*, *9*, 769. https://doi.org/10.3389/fpls.2018.00769

Li, X., & Brummer, E. C. (2012). Applied genetics and genomics in alfalfa breeding. *Agronomy*, *2*, 40–61. https://doi.org/10.3390/agronomy2010040

Li, X., Wei, Y., Acharya, A., Hansen, J. L., Crawford, J. L., Viands, D. R., Michaud, R., Claessens, A., & Brummer, E. C. (2015). Genomic prediction of biomass yield in two selection cycles of a tetraploid alfalfa breeding population. *Plant Genome*, *8*. https://doi.org/10.3835/plantgenome2014.12.0090

Madril, C. M., Pierce, C. A., & Ray, I. M. (2008). Heterosis among hybrids derived from genetically improved and unimproved alfalfa germplasm. *Crop Science*, *48*, 787–1792. https://doi.org/10.2135/cropsci2008.01.0050

Maruthavanan, J., Klypina, N., Sterling, T. M., & Ray, I. (2009, November). *Effect of progressive drought on yield, carbohydrate, and amino acid composition of different alfalfa accessions*. Paper 55774. Paper presented at the international annual meeting of the ASA, CCSA, and SSSA, Pittsburg, PA. http://a-c-s.confex.com/crops/2009am/webprogram/Paper55774.html

Maruthavanan, J., Ray, I., & Sterling, T. (2007). Physiological and biochemical responses of field grown alfalfa to water deficit. Proceedings: Western Society of Crop Science Annual Meetings, Las Cruces, NM. 18–19 June 2007. https://a-c-s.confex.com/crops/ws2007/techprogram/P35463.HTM

Melton, B., Currier, C., Miller, D., & Kimmell, J. (1989a). Registration of 'Malone' alfalfa. *Crop Science*, *29*, 485. https://doi.org/10.2135/cropsci1989.0011183X002900020050x

Melton, B., Miller, D., Teuber, L., & Walton, M. (1985). Registration of 'Dona Ana' alfalfa. *Crop Science*, *25*, 705. https://doi.org/10.2135/cropsci1985.0011183X002500040033x

Melton, B., Wilson, M., & Currier, C. (1989b). Registration of 'Wilson' alfalfa. *Crop Science*, *29*, 485-486. https://doi.org/10.2135/cropsci1989.0011183X002900020051x

Mir, R. R., Zaman-Allah, M., Sreenivasulu, N., Trethowan, R., & Varshney, R. K. (2012). Integrated genomics, physiology and breeding approaches for improving drought tolerance in crops. *Theoretical and Applied Genetics*, *125*, 625–645. https://doi.org/10.1007/s00122-012-1904-9

Mubako, S. T., & Lant, C. L. (2013). Agricultural virtual water trade and water footprint of U.S. states. *Annals of the Association of American Geographers*, *103*, 385–396. https://doi.org/10.1080/00045608.2013.756267

National Drought Mitigation Center. (2021). *U.S. drought monitor. Map archive*, *30 Nov. 2021*. https://droughtmonitor.unl.edu/Maps/MapArchive.aspx

Nepolean, T., Kaul, J., Mukri, G., & Mittal, S. (2018). Genomics-enabled next-generation breeding approaches for developing system-specific drought tolerant hybrids in maize. *Frontiers in Plant Science*, *9*, 361. https://doi.org/10.3389/fpls.2018.00361

Nyquist, W. E., & Baker, R. J. (1991). Estimation of heritability and prediction of selection response in plant populations. *Critical Reviews in Plant Sciences*, *10*, 235–322. https://doi.org/10.1080/07352689109382313

Orloff, S., & Putnam, D. (2015). Drought strategies for alfalfa. https://anrcatalog.ucanr.edu//pdf/8522.pdf

Ray, I. M., Han, Y., E, L., Meenach, C. D., Santantonio, N., Sledge, M. K., Pierce, C. A., Sterling, T. M., Kersey, R. K., Bhandari, H. S., & Monteros, M. J. (2015). Identification of QTL for alfalfa forage biomass productivity during drought stress. *Crop Science*, *55*, 2012–2033. https://doi.org/10.2135/cropsci2014.12.0840

Ray, I. M., Pierce, C. A., & Currier, C. G. (2012). Registration of 'NuMex Bill Melton' alfalfa for variable soil moisture environments. *Journal of Plant Registrations*, *6*, 137–140. https://doi.org/10.3198/jpr2011.10.0561crc

Ray, I. M., Segovia-Lerma, A., & Murray, L. W. (2004). Diallel analysis of carbon isotope discrimination and its association with forage yield among nine historically recognized alfalfa germplasms. *Crop Science*, *44*, 1970–1975. https://doi.org/10.2135/cropsci2004.1970

Ray, I. M., Townsend, M. S., Henning, J. A., Currier, C. G., & Melton, B. A. (1999c). Registration of NM-9D11A-PRR3 phytophthora resistant alfalfa germplasm with increased yield potential for irrigated and water-limited environments. *Crop Science*, *39*, 1537–1538. https://doi.org/10.2135/cropsci1999.0002rgp

Ray, I. M., Townsend, M. S., & Muncy, C. M. (1999b). Heritabilities and interrelationships of water-use efficiency and agronomic traits in irrigated alfalfa. *Crop Science*, *39*, 1088–1092. https://doi.org/10.2135/cropsci1999.0011183X003900040022x

Ray, I. M., Townsend, M. S., Muncy, C. M., & Henning, J. A. (1999a). Heritabilities and interrelationships of water-use efficiency and agronomic traits in water-stressed alfalfa. *Crop Science*, *39*, 494–498. https://doi.org/10.2135/cropsci1999.0011183X0039000200032x

Reynolds, M., & Tuberosa, R. (2008). Translational research impacting on crop productivity in drought-prone environments. *Current Opinion in Plant Biology*, *11*, 171–179. https://doi.org/10.1016/j.pbi.2008.02.005

Santantonio, N., Pierce, C. A., Steiner, R. L., & Ray, I. M. (2019). Genetic mapping of water use efficiency and carbon and nitrogen metabolism in drought-stressed alfalfa. *Crop Science*, *59*, 92–106. https://doi.org/10.2135/cropsci2018.05.0307

Sinha, P., Singh, V. K., Bohra, A., Kumar, A., Reif, J. C., & Varshney, R. K. (2021). Genomics and breeding innovations for enhancing genetic gain for climate resilience and nutrition traits. *Theoretical and Applied Genetics*, *134*, 1829–1843. https://doi.org/10.1007/s00122-021-03847-6

Sledge, M. K., Ray, I. M., & Jiang, G. (2005). An expressed sequence tag SSR map of tetraploid alfalfa. (*Medicago sativa* L.). *Theoretical and Applied Genetics*, *111*, 980–992. https://doi.org/10.1007/s00122-005-0038-8

Turner, N. C. (1981). Techniques and experimental approaches for the measurement of plant water status. *Plant and Soil*, *58*, 339–366. https://doi.org/10.1007/BF02180062

USDA–Economic Research Service. (2021). *Drought in the western United States*. https://www.ers.usda.gov/newsroom/trending-topics/drought-in-the-western-united-states/

USDA–National Agricultural Statistics Service. (2018). *2018 irrigation and water management survey*. https://www.nass.usda.gov/Publications/AgCensus/2017/Online_Resources/Farm_and_Ranch_Irrigation_Survey/fris.pdf

USDA–National Agricultural Statistics Service (2021). Crop production 2020 summary. https://downloads.usda.library.cornell.edu/usda-esmis/files/k3569432s/w3764081j/5712n018r/cropan21.pdf

Yu, L.-X. (2017). Identification of single-nucleotide polymorphic loci associated with biomass yield under water deficit in alfalfa (*Medicago sativa* L.) using genome-wide sequencing and association mapping. *Frontiers in Plant Science*, *8*, 1152. https://doi.org/10.3389/fpls.2017.01152

Yuegao, H., & Cash, D. (2009). Global status and development trends of alfalfa. In D. Cash, H. Yuegao, L. Kechang, W. Suqin, Z. Ping, & G. Rong (Eds.), *Capacity building in improved management of alfalfa in the southern mountainous areas of the Ningxia Hui autonomous region*. FAO. https://pdfs.semanticscholar.org/9608/88831ac693792ba8fa557e6ca32f362f864b.pdf

Zhang, T., Yu, L.-X., Zheng, P., Li, Y., Rivera, M., Main, D., & Greene, S. L. (2015). Identification of loci associated with drought resistance traits in heterozygous autotetraploid alfalfa (*Medicago sativa* L.) using genome-wide association studies with genotyping by sequencing. *Plos One*, *10*, e0138931. https://doi.org/10.1371/journal.pone.0138931

SUPPORTING INFORMATION

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