

*Full Length Research Paper*

# Mapping of QTLs for leaf area and the association with winter hardiness in fall-sown lentil

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Variations in plant architecture are often associated with the ability of plants to survive cold stress during winter. In studies of winter hardiness in lentil, it appeared that small leaf area was associated with improved winter survival. Based on this observation, the inheritance of leaf area and the relationship with winter hardiness using an  $F_6$  - derived recombinant inbred line (RIL) population from the cross of WA8649090 x Precoz was investigated. The WA8649090 parent was winter hardy and had small leaves, while the Precoz parent was non-winter hardy and had large leaves. The 106 RILs and the parents were planted in a field in a randomized complete block design with three replications. Leaf area ( $\text{cm}^2$ ) of leaves from the fourth node was measured using a flatbed scanner and WinRHIZO software. Average leaf area for WA8649090 was  $0.46 \text{ cm}^2$ , while leaf area for Precoz was  $0.89 \text{ cm}^2$ . Average leaf area of the RILs was  $0.63 \text{ cm}^2$ , and the frequency distribution was continuous, indicating the effects of more than one gene. Quantitative trait locus (QTL) analysis using a 130-point linkage map revealed one major QTL on linkage group 6 which explained 20.45% of the phenotypic variation for leaf area. The location of QTL for leaf area mapped the same region where one of the QTL for winter hardiness was mapped and significant association ( $r^2 = 0.750$ ,  $P < 0.01$ ) was found between leaf area and winter hardiness. These results indicated an association between winter hardiness and leaf area that provides information applicable to lentil breeding.

**Key words:** Leaf area, lentil, recombinant inbred line (RIL), QTL mapping, winter hardiness.

## INTRODUCTION

It has been of interest to find close relationships between two traits for use in selection to improve the trait that is more difficult. The first example of an application of this idea was practiced by Sax (1923) who investigated associations between a quantitative trait (seed size) and some other morphological traits (seed coat pattern and pigmentation).

Lentil leaves consist of one to 16 pair of leaflets and leaflet number may differ among the genotypes (Muehlbauer et al., 1995). Leaf morphology is in the form of alternate, compound and pinnate and leaves usually end with a tendril. First two nodes known as scale nodes develop below, or at the soil surface and may have

simple, scale like leaves. The first true leaves develop at the third node. Leaves at the third and fourth nodes are bifoliate, and the following ones are multifoliate (Saxena and Hawtin, 1981). Lentil seedlings can produce a new node every four to five days under good growing conditions.

Leaf characteristics e.g., leaf size, color, cell number and size, have long been explored and correlated with winter survival for use in indirect selection of winter hardy lines. Winter hardiness studies in wheat showed that leaf area in the fall was associated with winter hardiness (Klages, 1926). Genotypes with large leaf area, erect growth and tall in the fall, tend to be less tolerant to harsh winters and cold temperatures. Fowler et al. (1981) investigated associations between winter hardiness and chemical, anatomical, morphological or physiological characteristics in wheat and reported that the associations

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were inconsistent.

Significant associations were reported between winter hardiness and prostrate growth habit and anthocyanin pigmentation in lentil (Ali et al., 1999). In pea, anthocyanin pigmentation, purple flower color, prostrate growth habit and black-hilum were highly associated with winter hardiness (Markarian and Anderson, 1966; Anderson and Markarian, 1968; Liesenfeld et al., 1986). In wheat, winter survival was negatively correlated with total leaf fresh weight (Chun et al., 1998). Singh et al. (1989) studied the associations between cold tolerance and growth habit, leaflet area, days to flower, plant height and seed weight and reported that none of these traits could be used as selection criteria for cold tolerance in chickpea. Plant morphological traits such as growth type, leaf area and leaf type, were not well correlated with cold tolerance. However, these traits were considered important characters for winter survival in the field (Murray et al., 1988). It appeared from these studies that there were significant associations between winter hardiness and the traits mentioned earlier, but none of the factors mentioned earlier was used as consistent criteria for selection of winter hardy lines.

Molecular markers are valuable tools to locate genes responsible for quantitative traits in the genome. Investigating associations among traits of interest in terms of QTL mapping studies may provide information on the relationships between genes in the genome. A basic genetic understanding of these associations may help in the development of new breeding strategies for the improvement of winter hardy lentils. Quantitative trait loci studies on leaf area appear to be limited in legume crops. In soybean, Mansur et al. (1993) identified two QTLs for leaf size using a recombinant inbred line (RIL) population developed from Minsoy x Noir 1 cross. The QTL on LG 2 explained 20%, and the QTL on LG 16 explained 25% of the phenotypic variation for leaf size.

It has been observed that winter hardy lentils tend to have prostrate growth habit, small seed size, late flowering and late maturity (Erskine and Muehlbauer, 1995), while spring types have a more rapid growth rate, large seed, early flowering and early maturity. In the studies of winter hardiness in lentil, we observed associations between leaf area and winter hardiness. Therefore, the objectives of this study were to determine the inheritance of leaf area, identify genomic region(s) involved in the control of leaf area and determine the association between leaf area and winter hardiness if it could be applicable as selection criteria for winter hardiness in lentil breeding.

## MATERIALS AND METHODS

A RIL population developed from the cross of WA8649090 (winter hardy) x Precoz (non-winter hardy) was used to study leaf area. Winter hardy parent, WA8649090, had small leaves while susceptible parent, Precoz, had large leaves. 106 RILs together with the parents and checks (Brewer as susceptible and WA8649041 as

winter hardy check) were planted at Spillman Agricultural Experiment Station, Pullman, WA. The RILs together with parental lines were planted in a randomized complete block design with three replications. Plots were single rows, 1 m long and spaced 0.3 m apart with an average of 30 to 40 plants in each row.

Since it is difficult and time consuming to measure the entire leaf area of individual plants, leaf area was first measured at each node. Fall sown lentils at hardening stage usually develop 4 to 6 nodes (personal observations) and this corresponds to V4-V5 stages (Erskine et al., 1990).

To determine which leaflets at each node will provide better differentiation of RILs, parental lines and checks for leaf area at each node (from third to sixth nodes) was first compared. Leaf samples at each node were taken from 75 days-old fall-sown lentil plants and kept at 4°C in the refrigerator until they were measured. Leaf area of parents, checks and RILs were measured using a flatbed scanner (HP scanjet 6.9) and WinRHIZO 3.01 software (WinRHIZO, Regents Instruments Inc., 2001, Quebec, Canada). Average leaf area was determined by taking leaf samples from five plants in each plot and determined as cm<sup>2</sup>.

Data from recombinant inbred line population were analyzed using SAS software 8.0 (SAS, 2000) 'proc mixed' and 'proc glm' models. Inheritance of leaf area was determined based on frequency distribution for leaf area of the RIL population. Since F<sub>6</sub> derived RILs are expected to be nearly homozygous, discrete segregation of 1 to 1 would be expected for single gene inheritance. A continuous distribution pattern for leaf area would be indicative of more than one gene.

## Linkage mapping and QTL analysis

A genetic linkage map of lentil for winter hardiness studies was developed previously (Kahraman et al., 2004) and was used to map gene(s) responsible for leaf area. The details on the genetic map of this population were reported (Kahraman et al., 2004). In short, the genetic map was constructed with 130 polymorphic RAPD, ISSR and AFLP markers consisting of 9 linkage groups covering 973 cM.

QTL analysis was performed using Qgene3.0 (Nelson, 1997) and MapManager QT 2.8 (Manly and Olsen, 1999). Qgene was used for single point, interval QTL mapping and multiple regression analyses. MapManager QT was mainly used to check data quality such as erroneous double crossovers and to confirm the results generated by other programs. The percentage of the phenotypic variation (R<sup>2</sup>) explained by the detected QTLs was determined using multiple regression analysis by taking a marker at the peak of each QTL as factors. A LOD score of 2.0 was chosen as the threshold for declaring putative QTLs.

## RESULTS

### Genotypic variation for leaf area of parental lines and checks

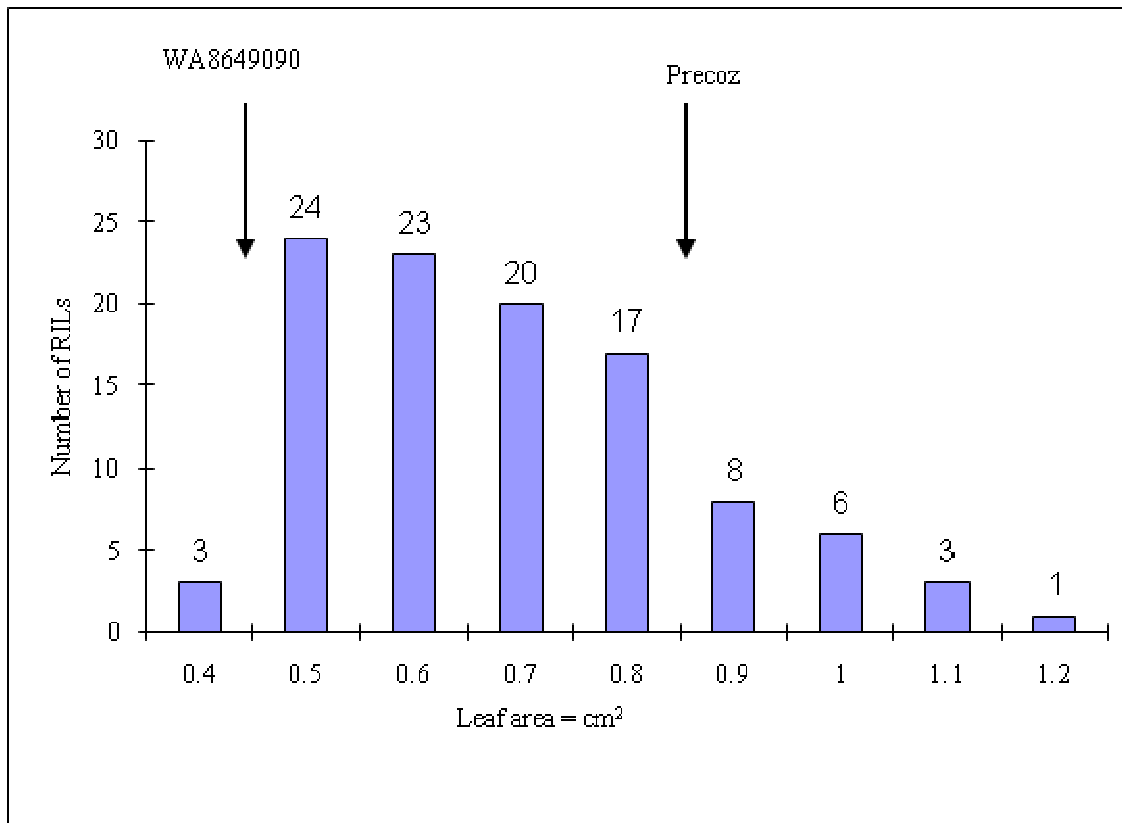
Fall grown lentil seedlings were developed from 4 to 6 nodes at hardening stage that corresponded to V4 and V5 stages. The first two nodes did not develop leaves. The third and fourth nodes developed bifoliate (a pair leaflet) leaves regardless of the genotypes, while the fifth and sixth nodes developed multifoliate leaves (from 2 to 4 leaflets) depending on the genotype's growth rate.

Total leaf area (leaf area of 3rd, 4th, 5th and 6th nodes) of spring cultivars (Precoz and Brewer) were significantly larger than the leaf area of the winter hardy

**Table 1.** Average leaf area at each node and total leaf area (leaflet area of node 3+4+5+6) of fall sown lentil genotypes at seedling stage.

Genotype	Average leaf area at each node (cm <sup>2</sup> )				
	3 <sup>rd</sup> Node	4 <sup>th</sup> Node	5 <sup>th</sup> Node	6 <sup>th</sup> Node	Total
Brewer	0.81 <sup>ax</sup>	0.93 <sup>a</sup>	1.07 <sup>a</sup>	0.64 <sup>a</sup>	3.45 <sup>a</sup>
Precoz	0.76 <sup>a</sup>	0.88 <sup>b</sup>	0.96 <sup>a</sup>	0.57 <sup>a</sup>	3.17 <sup>b</sup>
WA8649041	0.52 <sup>b</sup>	0.54 <sup>c</sup>	0.55 <sup>b</sup>	0.37 <sup>a</sup>	1.98 <sup>c</sup>
WA8649090	0.43 <sup>b</sup>	0.46 <sup>d</sup>	0.52 <sup>b</sup>	0.34 <sup>a</sup>	1.75 <sup>c</sup>
LSD <sub>0.05</sub>	0.050	0.04	0.14	0.49	0.06

\*Means with the same letter in each column are not significantly different from each other at P < 0.05.



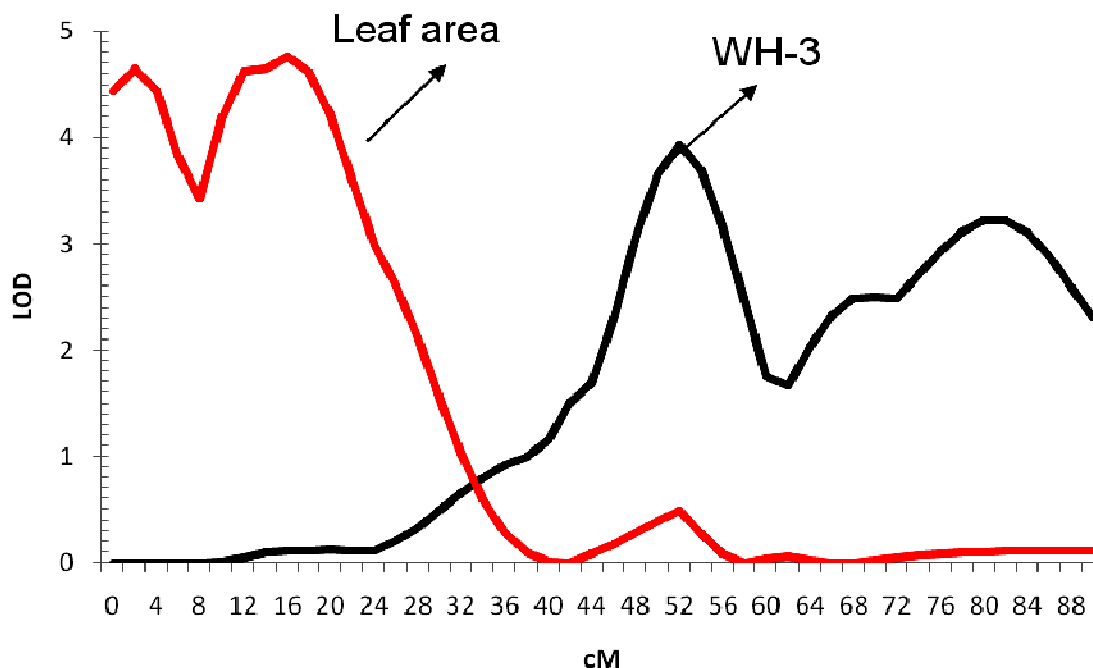
**Figure 1.** Frequency distribution of F<sub>6</sub> - derived RILs for leaf area at the fourth node from the cross of WA8649090/Precoz.

lines (WA8649090 and WA8649041). While leaf areas of the spring cultivars were significantly different from each other, leaf area of winter hardy genotypes was statistically not significant from each other. These results indicated the presence of genetic variation for leaf area in lentil at the seedling stage (Table 1).

When genotypes were compared for leaf area at each individual node, all genotypes were significantly different from one another at the fourth node (Table 1) indicating that leaf area at the 4th node was efficient in differentiating the genotypes among/within spring and winter types.

#### Genotypic variation for leaf area at the 4th node in RILs

Leaf area of the parents as well as the RILs differed significantly (P < 0.01). Average leaf area for the parents, WA8649090 and Precoz, at the 4th node was 0.46 and 0.88 cm<sup>2</sup>, respectively. Average leaf area of the RIL population was 0.63 cm<sup>2</sup> and ranged from 0.36 to 1.23 cm<sup>2</sup> (Figure 1). The RILs exhibited transgressive segregation for the leaf area. Frequency distribution for the leaf area was continuous and skewed toward winter hardy parent indicating quantitative inheritance of the trait.



**Figure 2.** QTL detected for leaf area and winter hardiness on linkage group 6 in lentil.

QTL analysis of leaf area revealed one major QTL (LOD = 4.6) on linkage group 6 which explained 20.45% of the phenotypic variation (Figure 2 and Table 1). The location of the QTL for leaf area mapped to the same region where one of the winter hardiness QTL was mapped and a significant association ( $r^2 = 0.750$ ,  $P < 0.01$ ) was found between leaf area and winter hardiness. Simple correlation and QTL analyzes results indicated an association of winter hardiness genes with genes for leaf area.

To find a recombinant type with large leaf area and high winter hardiness, each RIL was checked if any of them had large leaf area with high winter survival. With the available 106 RILs, none had the desired combination.

## DISCUSSION

Morphological characteristics of a plant are easily evaluated in breeding programs, and therefore their association with winter hardiness is of interest. This study in part was able to explain the presence of association between winter hardiness and leaf area.

Measurement of leaf area at the fourth node was effective in separating winter hardy lines from the non hardy ones and also separating more hardy ones from less hardy one. The methodology and equipment for the assessment of leaf area appears to be practical, efficient, user friendly, reliable and fast. Because it is difficult and time consuming to measure whole leaf area of individual plants when large number of progeny needs to be

screened for leaf size differences, quick, reliable and consistent method would save time and allow breeders to screen large progenies for leaf area differences to be able to select winter hardy types.

QTL mapping and simple correlation analyses in this study indicated some degree of association between winter hardiness and leaf area in lentil; however, the use of leaf area as a selectable marker for determining the winter hardy lentil genotypes appears to be questionable because QTL detected on LG6 for winter hardiness with a LOD score of 3.2 (Kahraman et al., 2004) and QTL for leaf area (LOD = 4.6) are not considered as major and strong QTL. While four QTL for winter hardiness were detected (Kahraman et al., 2004), only one QTL with small effect for winter hardiness was mapped to the same region where QTL for leaf area was mapped. Therefore, small leaf type might not be a requirement for winter hardiness but it might be required for high winter hardiness in lentil. Similar findings was reported by Klages (1926) who carried out winter hardiness studies in wheat, and he stated that leaf area is not a necessary indicator for winter hardiness, but it should be taken into account for the selection of winter hardy genotypes. In pea, average leaf area was negatively related to cold tolerance and when large leafed lines were removed from the populations, cold tolerance of genotypes within winter hardy germplasm could not be predicted based on leaf area (Acikgoz, 1982). Low correlations were reported between leaf traits and freezing tolerance in faba bean (Herzog, 1987).

The protective role of the morphological structure of a plant against low temperatures has long been a question

(Klages, 1926). It has often been reported that winter hardy cereals are mostly characterized by certain xerophytic structures such as narrow leaves and a prostrate growth habit (Klages, 1926). In studies of screening for winter hardiness in lentil germplasm, it was observed that small seed size was associated with winter hardiness (Erskine and Muehlbauer, 1995). Smaller size of organs, such as small leaf type, is considered advantageous for cold tolerance in grasses (Acikgoz, 1982). It can be assumed that smaller leaves are associated with smaller cells and that small cells are better at osmotic adjustment.

As it has been known that winter hardiness is controlled by many genes and influenced by many factors, it appeared difficult to determine close association between winter hardiness and leaf area. Leaf area of a genotype might also be related to seed size. Therefore, it would be interesting to investigate the associations among winter hardiness, leaf area and seed size in order to obtain new insights in terms of molecular analyses and develop new breeding strategies for development of new cultivars.

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