Heritability and Combining Ability for Cold Hardiness from Partial Dialelles in Iranian Pomegranate Cultivars

Ali Akbar Ghasemi Soloklui
Department of Horticultural Science, School of Agriculture, Shiraz University, Shiraz, Iran

Ali Gharaghani
Department of Horticultural Science, School of Agriculture, Shiraz University, Shiraz, Iran; and Drought Research Center, School of Agriculture, Shiraz University, Shiraz, Iran

Nuadzie Oraguzie
Department of Horticulture, Irrigated Agriculture, Research and Extension Center, Washington State University, 24106 N Bunn Road, Prosser, WA 99350

Armin Saed-Moucheshi
Department of Crop Science and Plant Breeding, School of Agriculture, Shiraz University, Shiraz, Iran

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Abstract. The development of cultivars with broader climatic adaptation has recently become the objective of most fruit breeding programmes. Regarding the importance of genetic control of cold hardness as an influential characteristic for pomegranate and lacking studies in this area, the genetic control of cold hardness in pomegranate using a partial mating scheme was studied. Five parents, including ‘Rabab Post Ghermez Neyriz’, ‘Malas Yazdi’, ‘Poost Sefid Dezful’, ‘Malas Pishva Varamin’, and ‘Poost Nazok Torosh Abarkuh’ with different cold hardness capability were screened following a cold hardiness test in the laboratory and an evaluation of cold injury after natural freezing events in the field. The five screened cultivars were crossed in half-diallel crossing scheme with a total of 10 crosses in the Spring of 2014. Cold hardness of the parent cultivars and the F1 progenies were investigated using the electrolyte leakage (EL) method. Results showed that both general combining ability (GCA) and specific combining ability (SCA) were statistically significant. The hardest parent (‘Poost Nazok Torosh Abarkuh’) showed the largest positive GCA effect (1560.59) for winter survival, suggesting that this parent is capable to produce tolerant offspring with high breeding values in crossing programs. The significant SCA in this study suggests that specific crosses should be targeted to produce highly capable offspring. Cross between ‘Poost Nazok Torosh Abarkuh’ and ‘Malas Pishva Varamin’ showed high value for SCA (1661.74), indicating capability for production of tolerant offspring to the cold condition. Furthermore, high broad-sense heritability (0.70) and moderate narrow-sense heritability (0.45) for cold hardness indicate that a reasonable progress could be made in improvement of this trait through conventional breeding.

Pomegranates are presumed to have originated in the Middle East (Persia, e.g., Iran) and Southeast Asia (including Turkmenistan and Afghanistan), areas with relatively cold winters and hot summers (Castle et al., 2011). Iran is considered to be one of the major producers (Holland et al., 2009). There is considerable interest in growing pomegranate trees in tropical, subtropical, and sub-tropical regions of the world, including southern Asia, Africa, and Europe, particularly, in countries such as Iran, India, Turkey, Afghanistan, Spain, Egypt, and other parts of North Africa, China, Italy, France, and the United States. Pomegranate consumption has increased recently because of studies that confirm that pomegranate may improve certain features of human health resulting from the high antioxidant content of its juice and peel and possession of properties which prevent cancer and cardiovascular diseases (Fuhrman and Aviram, 2006). Pomegranate is a natural out-cropper, the seeds germinate well and also is easily propagated by cutting (Jalikop and Kumar, 1990). It is widely distributed and have adapted to different environments. Thus, pomegranates display a wide diversity of pomological and tree characteristics. By using available germplasm, it is possible to develop cultivars with improved winterhardiness and acceptable fruit quality. Susceptibility to freezing injury is a major environmental factor limiting geographical distribution and growing season of many cultivars and affects crop quality and productivity (Thomashow, 1999). Fruit breeding efforts have recently focused on the development of cultivars with broader climatic adaptation (such as increased freezing tolerance for the northern regions), disease and pest resistance, and high fruit quality (Galletta and Ballington, 1996).

Cold hardiness is the result of complex physiological mechanisms involving many cellular and whole plant details. Moreover, winterhardiness is affected not only by tolerance to cold but also by tolerance to other factors such as frost, water logging, freeze-thaw cycles, and diseases (Steponkus, 1979). Knowledge of the hardiness of the genetic stock to winter freezing is critical to the success of tree fruit improvement programs and for selecting and breeding for improved cold hardiness. Cold hardness evaluation can be assessed by examining cold injury after natural frost events in field trials, although there are several limitations to screening methods relying on natural frost events for cold hardness measurement. First, field evaluation is typically established only for the short term on relatively mild, productive places which rarely receive damaging freezing. Second, the effects of infrequent frosts may be confounded with injury due to other causes. Third, the incidence, timing, and intensity of frosts are not uniform across the test place (O’Neill et al., 2001).

The evaluation of cold hardness under controlled conditions in a freezer (artificial freezing testing) is a common test to assay plant tissue samples for cold injury (Burr et al., 1990). In this method, simple and inexpensive measurement of cold hardness can be carried out for large number of plant samples (O’Neill et al., 2001). Ghasemi Soloklui et al. (2012) reported that EL measurement and tetrazolium stain test after controlled freezing allowed them to discriminate among pomegranate cultivars for freezing tolerance. O’Neill et al. (2001) reported that assaying for cold hardness at the seedling stage has advantages over testing an adult tree. The seedling tests require less space and time than field assays and provide more uniform test conditions, resulting in a greater statistical precision and cost-effectiveness.

Earlier studies have shown that the genetic control of cold hardiness is very complex and mostly polygenic (Snape et al., 1997). Generation means analysis revealed that cold hardiness is a quantitative trait controlled largely by additive gene effects and, to a lesser extent, the dominance gene effects, with cold sensitivity appearing to be...
Numerous studies have shown that cold hardiness is inherited in an additive manner and progenies are intermediate between the parents, in both herbaceous and woody crops (Tang, 2002). Cold hardiness could be explained adequately by a simple additive–dominance model of gene function, although two epistatic models involving additive–additive and dominance–dominance interactions also fit the data (Arora et al., 2000).

Genetic studies on winterhardiness of pomegranate are limited. A review of the literature on temperate fruit crops shows that heritability estimates have been generated for several aspects of freezing tolerance in a few fruit crops (including plum, peach, blueberry, and apple) and the values reported are moderate to high (Arora et al., 1992; Ashworth and Wisniewski, 1991; Howe et al., 2003; Luby, 1991; Quaamme, 1978). Also, genetic components of the variance have not frequently been obtained for freezing tolerance in fruit tree, but additive variance has been reported to be of major importance in apple and blueberry (Fear et al., 1985; Watkins and Spangelo, 1970). The moderate to high levels of heritability of cold hardness in fruit tree suggest that identifying cold hardy cultivars in the germplasm and transferring that property to progenies is not difficult, but rather the combination of multiple quantitative traits including cold hardness and fruit quality is the major challenge (Owens, 2005).

This study aimed to assess the level of genetic control of cold hardness of the F1 progeny resulting from crosses between pomegranate cultivars with varied degrees of cold hardness. Specific objectives include 1) estimating genetic parameters, including broad- and narrow-sense heritabilities and general and specific combining abilities, 2) prediction of breeding values and determination of potential genetic gain as well as genetic correlations between pairs of traits.

Materials and Methods

Plant material. Five parent cultivars, including Rabab Post Ghermez Neyriz, Malas Yazdi, Poost Sefid Dezful, Malas Pishva Varamin, and Poost Nazok Torosh Abarkuh were used in the hybridization program (Table 1). These cultivars were selected based on pervious cold hardness tests in the laboratory and cold injury evaluations after natural frost events in the field. The parents were crossed using a half-diallel crossing scheme excluding reciprocals involving the population of each parent cultivar. The F1 progeny and their corresponding F2 progenies were collected and propagated. The F1 progeny was propagated from the parent plants and the F1 seeds were propagated by cuttings.

Cold hardiness measurement. One-year-old shoots (10 cm length) of axially propagated parent plants and the F1 seedlings were collected on 16 Jan. 2015. The samples were wrapped in a wet paper, sealed in plastic bags, and kept at 5 °C during transportation from the field to the laboratory. The shoots were washed with deionized water. One-centimeter-long segments were cut and placed in 50-ml plastic tubes. One milliliter of deionized water was added to each sample for immediate ice formation. The tubes were transferred to a freezing chamber and exposed to low temperatures. The cooling rate was 2 °C·h⁻¹, whereas freezing treatments were -6, -9, -12, -15, -18, -21, -24, -27, and -30 °C. The samples were kept at given treatment temperatures for 1 h and then transferred to 5 °C, withdrawn and thawed. Twenty milliliters of distilled water was added to tubes and shaken for 1 h at 23 °C. The tubes were kept at room temperature for 24 h before the first electrical conductivity (EC1) measurement. The samples were autoclaved for 20 min to allow maximum leakage of ions, cooled at room temperature, and again EC was measured (EC2). The relative EL was calculated as REL = (EC1/EC2) × 100. Cold hardness was expressed as LT50 (lethal temperature at which 50% of the ion leakage occurs), the lethal temperature at which 50% of the tissues are dead) by fitting the response curves with the following logistic sigmoid function (Ghasemi Soloklui et al., 2012):

\[ \Delta R = \frac{a}{1 + e^{b(x-c)}} + d, \]

where \( x \) = treatment temperature; \( b \) = slope at inflection point; and \( c, a, \) and \( d \) determine the asymptotes of the function.

Statistical analyses. The diallele crosses were analyzed following the model of Griffing (1956) for the first trial and the partial diallele model for the second trial (Kempthorne and Curnow, 1961). Student’s \( t \) tests were performed to determine the significance of the estimated genetic parameters, and genetic components estimated to be different from zero at \( P < 0.05 \) were considered to contribute significantly to the model. All analyses were carried out using R 3.4.2 software. Diallele analysis according to the used models was conducted by the “DialleleAnalysisR” package downloaded from the R main repository (https://r-project.org/) and the codes were based on the guidance of the package.

Table 1. Origin, fruit, and tree characteristics of the pomegranate cultivars used in this study.

| Cultivar/ancestor | Province     | Fruit and tree characteristics                      |
|------------------|--------------|-----------------------------------------------------|
| Poost Nazok Torosh Abarkuh | Yazdi         | Medium fruit, red skin and aril, sweet-sour taste, locally important |
| Poost Sefid Dezful  | Khuzestan    | Small fruit, yellow-white skin, sweet-sour taste, white aril, locally important |
| Malas Yazdi       | Yazdi         | Big fruit, red skin and aril, sweet-sour taste, commercial cultivar |
| Rabab Poost Ghermez Neyriz | Fars       | Big fruit, red skin and aril, sweet-sour taste, commercial cultivar |
| Malas Pishva Varamin | Tehran    | Medium fruit, yellow skin, white aril, sweet taste, locally important |
Results and Discussion

For diallele data analysis using both Hayman and Griffing methods, the mean value matrix of cold hardiness for all crosses combinations was used (Table 2). Based on the equation for cold hardiness and the mean values (Table 2), the cold hardiness of all genotypes was negative. Analysis of variance (ANOVA) based on a complete block design (Table 3) revealed that the differences among the genotypes were significant ($P < 0.0001$).

Cold hardiness for the parental cultivars ranged from $-24.25$ °C (‘Poost Nazok Torosh Abarkuh’) to $-22.55$ °C (‘Malas Pishva Varamin’), whereas cold hardiness for the $F_1$ population ranged from $-23.19$ to $-19.42$ °C. Arora et al. (2000) showed that freezing (CSR parent) and $-12$ to $-14.3$ °C (drw parent) and freezing tolerance distributions for the blueberry parental populations ranged from $-19.8$ to $-21.3$ °C (csr parent) and $-12$ to $-14.3$ °C (drw parent).

Also, GCA and SCA (Levitt and Scarth, 1936) appeared to be significant according to ANOVA (Table 3). Cilas et al. (2003) in a diallele study reported that additive trait transmission with highly significant GCA effects is always greater than the SCA effects. This means that parental lines, or at least one of those, have a high GCA and a high potential to cross with other lines to generate future generations. Furthermore, the significant SCA effects showed that at least two pairs of parental lines have a high ability to be crossed with each other to produce offspring with a high breeding value. Using expected values for mean squares in the ANOVA (Table 3), the additive and dominance effects of the gene or genes controlling cold hardiness were estimated. The results showed that the genes controlling cold hardiness are influenced by both additive and dominance effects, although dominance effects were higher. This result suggests that breeding for cold hardiness entails selecting the best parental lines for use in crosses to capture both the dominance and additive effects of these genes.

Many studies have shown that cold hardiness is inherited in an additive manner, with progenies being intermediate between the parents, in both herbaceous and woody plants (Hummel et al., 1982; Tibbits et al., 1991).

To understand the combining ability of the parental lines crossed with one another, the Griffing method was used to estimate both GCA and SCA effect of each parental line (Table 4). ‘Poost Nazok Torosh Abarkuh’ and ‘Rabab Post Ghmernez Neyriz’ showed high positive GCA values, with the GCA of ‘Poost Nazok Torosh Abarkuh’ being the highest. This suggests that ‘Poost Nazok Torosh Abarkuh’ has a high ability to be crossed with all cultivars to produce the desired offspring for the next generation. On the other hand, ‘Malas Yazdi’, ‘Poost Sefid Dezful’, and ‘Malas Pishva Varamin’ showed negative GCA, indicating a low GCA of these cultivars for cold hardiness. The significant GCA indicates that parents that combine well with several other parents may provide opportunities for breeding improved winterhardiness. A nonhardy parent including ‘Malas Pishva Varamin’ had the largest negative GCA effect (–1390.67), whereas the hardest parent ‘Poost Nazok Torosh Abarkuh’ had the largest positive GCA effect (1560.59) for winter survival, indicating that this parent would be the best choice to use as the parent in crosses for cold hardiness.

Griffing (1956) reported analysis of diallele crosses that partitions the total genetic variation into GCA of the parents and SCA of the crosses. GCA is the average performance of a particular inbred in a series of hybrid combinations, whereas SCA refers to the performance of a combination of specific inbreds in a particular cross (Sprague and Tatum, 1942). Falconer (1975) reported that the GCA and SCA variances are largely due to additive and nonadditive gene actions, respectively. Moreover, Machikowa et al. (2011) suggested that combining ability is used in understanding the nature of gene action involved in the expression of quantitative traits and to predict the performance of the progenies.

The SCA for pairs of parental lines (Table 4) showed that the cross between ‘Poost Nazok Torosh Abarkuh’ and ‘Malas Pishva Varamin’ could produce seedlings with high SCA in the next generation [the SCA was positively high (1661.735), although the GCA of ‘Malas Pishva Varamin’ was low]. ‘Poost Nazok Torosh Abarkuh’ also showed positive SCA with ‘Poost Sefid Dezful’ and ‘Malas Yazdi’; the SCA for a cross between ‘Rabab Post Ghmernez Neyriz’ and ‘Poost Sefid Dezful’ as well as with ‘Rabab Post Ghmernez Neyriz’ was also positively high. All other SCAs for crosses between parental lines were negative, indicating poor performance in the progenies that will result if these crosses were made for breeding purposes.

Table 2. Mean of the crosses and the parental lines in relation to cold hardiness in pomegranate.

|            | Poost Nazok Torosh Abarkuh | Poost Sefid Dezful | Malas Yazdi | Rabab Poost Ghmernez Neyriz | Malas Pishva Varamin |
|------------|----------------------------|-------------------|-------------|-----------------------------|---------------------|
| Poost Nazok Torosh Abarkuh | –24.25 | –21.29 | –22.04 | –20.37 | –22.49 |
| Poost Sefid Dezful           | –19.20 | –19.42 | –19.52 | –23.19 | –22.55 |
| Malas Yazdi                  | –20.00 | –21.5  | –23.03 | –16.70 | –19.60 |
| Rabab Poost Ghmernez Neyriz  | –1,390.67 | 1,667.70 | –2,531.68 | 0.0000 | 0.0000 |
| Malas Pishva Varamin         | –517.04 | –3,405.32 | 1,082.45 | –2,156.69 | 0.0000 |

Table 3. Analysis of variance and genetic parameters for cold hardiness in pomegranate.

|            | DF | SS   | MS   | F   | P     |
|------------|----|------|------|-----|-------|
| Block      | 3  | 5.44 | 1.813| 0.791| 0.056 |
| Genotype   | 14 | 1,345.02 | 96.072| 41.92 | 0.000 |
| GCA        | 4  | 595.01 | 148.752| 64.906| 0.000 |
| SCA        | 10 | 750.04 | 75.004| 32.727| 0.000 |
| Error      | 694 | 1,590,498 | 2,292 | 0.000 |

Table 4. General combining ability (diagonal) and specific combining ability (above the diagonal) of cold hardiness for all parental pomegranate cultivars.

|            | Poost Nazok Torosh Abarkuh | Poost Sefid Dezful | Malas Yazdi | Rabab Poost Ghmernez Neyriz | Malas Pishva Varamin |
|------------|----------------------------|-------------------|-------------|-----------------------------|---------------------|
| Poost Nazok Torosh Abarkuh | 1,560.59 | 473.86 | 295.60 | –774.05 | 1,661.74 |
| Poost Sefid Dezful           | –517.04 | –3,405.32 | 1,082.45 | –2,156.69 | 0.0000 |
| Malas Yazdi                  | –142.04 | 1,667.70 | 2,531.68 | 0.0000 |
| Rabab Poost Ghmernez Neyriz  | 489.16  | –3,162.88 | –1,390.67 | 0.0000 |
| Malas Pishva Varamin         | –517.04 | –3,405.32 | 1,082.45 | –2,156.69 |

Table 5. Genetic parameters estimated using Hayman’s method for diallele mating.

| Parameters                                      | Value | Student’s t/test | P value |
|------------------------------------------------|-------|-----------------|---------|
| Additive effects (D)                           | 5.56  | 1.21            | 0.035   |
| Dominance effects (H1)                         | 3.99  | 3.23            | 0.000   |
| Dominance effects (H2)                         | 3.14  | 2.87            | 0.001   |
| F                                              | –4.76 | –0.42           | 0.420   |
| Dominance mean ratio                           | 0.84  |                 |         |
| Dominance and recessive allele’s frequency ratio in parental lines | 0.20 |                 |         |
| Additive and reductive gene ratio              | 0.26  |                 |         |
| Number of gene group                           | 0.57  |                 |         |
| Dominance and recessive genes ratio            | 1.04  |                 |         |
| Narrow-sense heritability                      | 0.45  |                 |         |
| Broad-sense heritability                       | 0.70  |                 |         |
freezing resistance in eucalyptus families. Tibbits et al. (1991) estimates that this trait particularly in the studied plant advances can be made through breeding for it (0.45) of cold hardiness suggest that moderate narrow-sense heritability and polypeptides of bark and xylem tissues. Plant Physiol. 100:690–696.

Arora, R., M.E. Wisniewski, and R. Scorza. 1992. Cold acclimation in genetically related (sibling) deciduous and evergreen peach (Prunus persica [L.] Batsch) I. Seasonal changes in cold hardiness and polypeptides of bark and xylem tissues. Plant Physiol. 99:1562–1568.

Ashworth, E. and M. Wisniewski. 1991. Response of fruit tree tissues to freezing temperatures. HortScience 26:501–504.

diallele crosses. Both additive effects (D) and nonadditive effects such as dominance (H1 and H2) were statistically significant (Table 5). These results are similar to the results obtained using Griffing’s method, which demonstrate that gene effects for cold hardness in pomegranate are both additive and dominant. Both H1–H2 that resulted in a positive value and the ratio of H2/4H1 (0.20) indicate that alleles for cold hardness genes in the parental lines are not equal. The F–F was not significant, suggesting that the additive effect genes are higher in frequency than genes with reductive effects. The ratio of dominance/recessive genes was ≈1, showing the effects of recessive and dominance genes for cold hardiness. This result is similar to D, H1, and H2 results.

Dominance mean ratio (0.84) was lower than 1 but higher than 0.5, suggesting that partial dominance is controlling cold hardiness rather than complete dominance. Watkins and Spangelo (1970) suggested that dominance and epistasis were not the major factors controlling cold hardness in apple (Malus sp.), instead low temperature–induced bud damage was controlled by additive gene action. Also, Tibbits et al. (1991) reported that freezing tolerance was inherited in a predominantly additive manner in interspecific hybrids. Research in other genera, including Solanum (Stone et al., 1993) and Rhododendron (Linn et al., 1998), suggested oligogenic control for cold hardness.

Relatively high broad-sense heritability (0.70) and moderate narrow-sense heritability (0.45) of cold hardness suggest that advances can be made through breeding for this trait particularly in the studied plant materials. Tibbits et al. (1991) estimates that individual narrow-sense heritability for freezing resistance in eucalyptus families was $h^2 = 0.66$ to 0.46. High narrow-sense heritability indicates that additive genetic effects are high and breeders could exploit the additivity of the genes controlling cold hardiness to improve this trait in their breeding programs. Numerous studies showed varied heritability for cold hardiness in fruit crops such as peach (midhigh), plum (>0.0), blueberry (high), and apple (midhigh) (Arora et al., 1992; Ashworth and Wisniewski, 1991; Howe et al., 2003; Luby, 1991; Quamme, 1978). It will be worthy to mention that all that is known regarding the inheritance of freezing tolerance in many fruit crops is simply that the trait is quantitatively inherited and that cold hardness can be transferred to the progeny.

Graphical analysis of these results using Hayman’s method based on variance and covariance of the parental lines (Fig. 1) showed that the intercept of the regression line is higher than zero. This indicates that dominance effect for cold hardiness in pomegranate is not complete, neither is it over-dominant. This result agrees with the dominance mean ratio discussed before. ‘Poost Nazok Torosh Abarkuh’ is located at the starting point of the regression line, whereas the ‘Malas Yazdi’ is located at the end of the line. ‘Rabab Poost Ghermez Neyriz,’ ‘Malas Pishva Varamin,’ and ‘Poost Sefid Dezful’ that are distant from one another are placed in the second of regression line near the end. These results showed that ‘Poost Nazok Torosh Abarkuh’ is the parental line with the highest dominance effect than other cultivars, whereas ‘Malas Yazdi’ showed the least dominance effect. Juxtaposing these results with the result from Griffing’s method for calculating SCA, where ‘Poost Nazok Torosh Abarkuh’ and ‘Malas Pishva Varamin’ showed the highest SCA, we would suggest selecting these two cultivars as the best candidate pair to cross in future to improve cold hardiness and to also generate a highly diverse progeny population for new cultivar development. The other cultivars seem to show partial or incomplete dominance in comparison with these two cultivars.

**Conclusion**

The high broad-sense heritability and narrow-sense heritability of cold hardness suggest that there is high potential for cold hardness improvement in pomegranate cultivars through conventional breeding programs. The hardiest parent ‘Poost Nazok Torosh Abarkuh’ had the largest positive GCA effects, whereas the highest SCA was recorded in a cross between ‘Poost Nazok Torosh Abarkuh’ and ‘Malas Pishva Varamin’, although the GCA of ‘Malas Pishva Varamin’ was low. This finding confirms that ‘Poost Nazok Torosh Abarkuh’ would be the best choice to include as a parent in breeding programs for development of new cold hardy cultivars.

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**Fig. 1.** Graphical analysis using Hayman’s method based on variance (Vr) and covariance (Wr) of parental cultivars in the horizontal and vertical axes (straight line: regression line; curve: confidence level).
