RESEARCH NOTE

Inheritance of carthamin and carthamidin in safflower (*Carthamus tinctorius* L.)

Pooran Golkar*

Research Institute for Biotechnology and Bioengineering, Isfahan University of Technology, Isfahan, Iran

Email*: golkar@cc.iut.ac.ir

Running title: Inheritance of carthamin and carthamidin

Keywords: epistasis; gene effect; heritability; pigment.

Introduction

Safflower (*Carthamus tinctorius* L.) is an important edible oilseed crop belongs to the family of *Asteraceae* (Singh 2007, Al-Snafi 2015). Safflower is cultivated over different geographical regions of Asia, including China and India (Singh 2007). Iran is considered as one of the major origins of safflower in the old world (Golkar 2014). It is a multipurpose crop used for its edible oil as well as its industrial, ornamental and medicinal applications (Uher 2008; Hussain *et al*. 2016). Traditionally, it was first grown for the pigment of flowers to color foods and dye clothes (Sultana and Anwer 2014). Easy collection of safflower and its remarkable biological activities have turned this plant to both a food and a medicine in many parts of the world (Asgarpanah and Kazemivash 2013). *C. tinctorius* has recently been shown to have antioxidant, anti-inflammatory and antidiabetic activities (Asgarpanah and Kazemivash 2013).

The most important characteristics indicating the coloring properties value in safflower are the contents of carthamin and carthamidin pigments in flower (Machewad *et al*. 2012; Asgarpanah and Kazemivash 2013). The safflower pigments including red (carthamin) (3-6%), which is insoluble in water, and yellow (carthamidin) (24-30%), which is soluble in water (Machewad *et al*. 2012). The safflower pigments are employed for various applications such as natural food colorant (Yue *et al*. 2013; Al-Snafi 2015).

Historically, safflower was cultivated as a source of a valuable carthamin whose glycoside was readily soluble in alkalis and alcohol (Yue *et al*. 2013). Eco-friendly and biodegradable dyes derived from natural resources have emerged as an important alternative to the synthetic dyes (Jadhav and Joshi 2015). Carthamidin is utilized in food products to prevent the petal waste, to serve as an artificial color, and to improve the therapeutic value of food products (Machewad *et al*. 2012; Al-Snafi 2015). Safflower carthamin is widely used as stain coloring in foods such as ice-cream, jelly and soup, and as an additive in beverages and cosmetics (Singh 2007, Machewad *et al*. 2012). China has manufactured and produced carthamin as a red paint for cosmetics (Yue *et al*. 2013).

The extracts of florets are used in the treatment of many illnesses such as menstrual problems, cardio vascular diseases pain, and swelling associated with trauma, heart attack and renal thrombosis (Singh 2007). In recent years, safflower petals have been used as herbal tea in India and China (Sultana and Anwer 2014; Al-Snafi 2015). Carthamin, which is extracted from safflower petals, is used for the treatment, in the form of infusion, of circulatory system disorders (Singh 2007).
The medicinal properties of safflower have been widely accepted; this is why there has been a growing demand for safflower petals. Hence, given the economic importance of safflower petals, it could be worthwhile to explore these florets for the extraction of colorants that would be used in different food products with medicinal properties. Improvement in the chemical characteristics of ice cream is reported by the addition of carthamidin extract (Machewad et al. 2012).

The literature review shows that there is no study addressing pigments (carthamin and carthamidin) enhancement in the petals of flowers via genetic based breeding methods. For the genetic improvement of safflower florets, the breeding method adopted mainly depends on the nature of gene action involved in the expression of the quantitative trait of safflower pigments. The majority of the flower-related traits are qualitative under simple genetic control. Studies of carthamin variation are rare in the case of safflower (Jadhav and Joshi 2015). The inheritance of flower color has been studied in safflower (Golkar et al. 2010; Leus 2016), but the inheritance of safflower pigments as a quantitative value has not been reported yet.

Generation mean analysis is a simple but useful technique used to estimate gene effects for a polygenic trait (Kearsey and Pooni 2004; Nakhaei et al. 2014). Its greatest merit lies in the ability to estimate epistatic gene effects, such as additive × additive, dominance × dominance and additive × dominance effects (Kearsey and Pooni 2004; Viana 2008). The presence or absence of epistasis can be detected by the analysis of generation means using the joint scaling test, which measures epistasis accurately, no matter if it is complimentary (additive × additive: i) or duplicate (additive × dominance: j) and (dominance × dominance: l), at the digenic level (Kearsey and Pooni 2004).

Therefore, the objective of this study was identification of the inheritance mode and the number of genes controlling carthamin and carthamidin in a population consisting of a hybrid from two completely different genotypes; this was meant to provide a basis for the evaluation of selection methods employed for the improvement of the mentioned pigments in safflower populations.

Keywords: epistasis; gene effect; heritability; pigment.

Materials and Methods

Plant materials and generations production

In this study, three genotypes consisting of two genotypes selected from Iranian local populations (White flower-Esfahan and C111) and a Mexican variety (2-138) were used as the parents. The genotypes of Wht – Esf and C111 were spininess with white and yellow flowers, respectively. The Mex. 2-138 genotype was a Mexican one with red flowers and a spiny character. The experiments involved the six basic generations: P1 and P2 parent cultivars, F1 and F2 first and second filial generations, and BC1 and BC2 (first and second back crosses) of the combinations of the parental cultivars. The genotypes of Mex.2-138 and C111 were considered as P1 and the genotype of Wht- Esf was taken as P2, in both crosses. The crosses between parental genotypes were made artificially by hands in spring, 2015. F2 seeds of the crosses were produced by bagging F1 plants in paper bags prior to the flowering period in summer, 2015. The generations of BC1 and BC2 of two crosses were produced by crossing the F1 plants with the parental genotypes of Mex. 2-138 and C111 (as P1) and the genotype of Wht – Esf (as P2) in two crosses, respectively. We used the parents of the respective cross as the male parent and the F1 generation as the female parent and the affected back crosses to produce BC1 and BC2 generations. The segregating (BC1, BC2 and F2) and non-segregating populations (P1, P2 and F1) were cultivated in a randomized block design with two replications at the Research Farm of Isfahan University of Technology, Isfahan, Iran, in spring, 2016. The number of row-length was three meters, but the number of rows varied: 2 row, for P1, P2 and F1; 4 rows for F2; and 3 rows for BC1 and BC2 in each replication. The sample size (i.e. the number of plants analyzed) varied: 10 plants for P1, P2...
and F₁ generations; 45 plants for F₂ generations; and 30 plants in BC₁ and BC₂ generations in each replication. The carthamin and carthamidin content were measured at physiological ripening for each plant in different generations.

**Carthamin extraction:**

Extraction of carthamin from safflower florets was essentially carried out by Fatahi et al. (2008), with some modifications mentioned here. Fine dry florets (1 g) were suspended in 20 ml of 0.5% (W/V) sodium carbonate and stirred at room temperature for 30 min. The floating pieces of dry florets were removed by centrifugation at 3500 rpm for 15 min, in a Hettich centrifuge (Universal 320R model), and the supernatant was retained at 4±1 °C. The resulting suspension was added to the fresh 20 ml 0.5% sodium carbonate and stirred for 30 min and centrifuged. This process was repeated two times. The cooled extracts were mixed and acidified to obtain the pH=3.5 by adding 0.5% citric acid; then they were used for the adsorption of carthamin. Adsorption of carthamin from the acid extract was performed using cellulose powder (Sigma-Aldrich), according to Fatahi et al. (2008), with some minor modifications. Cellulose powder (0.5 g) was suspended in acid solution (citric acid 0.5%), stirred by a magnetic stirrer on a hot plate (IKA model) at room temperature for 30 min, and then again centrifuged at 3500 rpm for 15 min. After centrifuging, the supernatant was discarded. Then the pellet was resuspended in distilled water and centrifuged. After centrifuging, the supernatant was discarded and the remaining pellet of each sample was freeze dried at -45 °C by a Freeze Dryer (Christ Com. Model, Alpha 1-2LD plus) for 18 h (Machewad et al., 2012). After that, the totally dried pellet was suspended in 10 ml of acetone and intermixed for 5 min at 3500 rpm in the centrifuge. The acetone layer was filtered and used for spectrophotometric (Unico, UV-2100) measurement. The spectrophotometric measurement of carthamin varied from 380 to 520 nm, and the absorbance value (or optical density value) ranged from 0.1 to 1.30.

**Carthamidin extraction**

One gram of safflower petals was soaked in 15 ml of distilled water for 2 h and stirred at 40 °C for 30 min, this procedure was repeated 2-3 times. Floating pieces were removed by centrifuging at 3500 rpm for 15 min and the supernatant was retained at 5 ±1 °C. The resultant suspension in distilled water was stirred for further 30 min and centrifuged. The supernatant was then filtered with the 200 (μ) mesh to separate the suspended particles of the powder from solution and then the filtered solution was concentrated with the rotary evaporator. The pellet of all samples was frozen at -45 °C by the Freeze Dryer. After that, the totally dried pellets were suspended in 10 ml of acetone and intermixed for 5 min at 3500 rpm in the centrifuge. The acetone layer was used for the spectrophotometric (Unico, UV-2100) measurement. The spectrophotometric measurement of carthamidin was in the range of 383-620 nm for the safflower yellow. The absorbance value for carthamidin (or OD value) ranged from 0.2 to 1.72.

**Statistical analysis:**

The generation mean analysis was performed according to Mather and Jinks (1982) to estimate the genetic components of variation, epistasis and gene effects in two steps: (i) testing for epistasis to determine the presence or absence of interallelic interaction, and (ii) estimating gene effects, variances and the type of epistasis involved. The generation mean was calculated as given (Mather and Jinks, 1982); \( P₁ = m + (d) \); \( P₂ = m − (d) \); \( F₁ = m + (a) \); \( F₂ = m + 1/2 (a) \); \( BC₁ = m + 1/2 (a) + 1/2 (d) \); \( BC₂ = m − 1/2 (a) + 1/2 (d) \).

Scaling test for A, B, C and D scales was applied to test the adequacy of simple additive – dominance model (Mather and Jinks, 1982). The values of A, B, C and D scales were constructed using the following formulae:

\[
A = 2BC₁ − P₁ − F₁; B = 2BC₂ − P₂ − F₁; C = 4F₂ − 2F₁ − P₁ − P₂; D = 2F₂ − BC₁ − BC₂.
\]

The variances of A, B, C and D scales were computed as follows: \( Vₐ = 4VBC₁ + VP₁ + VF₁ \); \( Vₐ = 4VBC₂ + VP₂ + VF₁ \); \( Vₐ = 16VF₂ + 4VF₁ + VP₁ + VP₂ \).
\( V_D = 4V_{F_2} + V_{BC_1} + V_{BC_2} \). The standard errors of A, B, C and D were obtained and utilized for testing the significance of the deviations of the respective scales from zero.

The joint scaling test (Mather and Jinks 1982) was employed to estimate the mean \((m)\), additive effect \((d)\), dominance effect \((h)\), additive \(\times\) additive \((i)\), additive \(\times\) dominance \((j)\) and dominance \(\times\) dominance \((l)\) parameters according to the following formulae: \( Y = m + \alpha[d] + \beta[h] + \alpha^2[i] + 2\alpha\beta[j] + \beta^2[l] \), assuming that there were no linkage and no higher order gene interaction. In this equation, \( \alpha, \beta, \alpha^2, 2\alpha\beta, \text{ and } \beta^2 \) are the coefficients for the genetic parameters of \([d], [h], [i], [j] \text{ and } [l] \), respectively. Accordingly, by the least squares computation method, the following formulae were used for arriving at different gene effects.

Mean \( = m = F_{2} \); additive effect \( = (a) = BC_1 - BC_2 \); dominance effect \( = (D) = 2BC_1 + 2BC_2 + F_1 - 4F_2 - 1/2P_1 - 1/2P_2 \); additive \(\times\) additive epistatic effect \( = (i) = 2BC_1 + 2BC_2 - 4F_2 \); additive \(\times\) dominance epistatic effect \( = (j) = BC_1 - 1/2P_1 - BC_2 + 1/2P_2 \); dominance \(\times\) dominance interaction effect \( = (l) = P_1 + P_2 + 2F_1 + 4F_2 - 4BC_1 - 4BC_2 \). The best fit model was the one which had significant estimates of all parameters along with non-significant chi-square value (Mather and Jinks, 1982).

The variance components for the six generations were calculated through the formula developed by Mather and Jinks (1982) as:

\[
D = 4V_{F_2} - 2(V_{BC_1} + V_{BC_2}), \\
H = 4 (V_{BC_1} + V_{BC_2} - V_{F_2} - V_{P_1}), \\
E_w = (V_{P_1} + V_{P_2} + 2V_{F_2})/4 \\
F = V_{BC_1} - V_{BC_2}, \\
V_T = V (F_{2}).\]

Here, \( V_{F_2}, V_{T}, V_{BC_1}, V_{BC_2}, V_{P_1} \text{ and } V_{P_2} \) are variances of \( F_2, F_1, BC_1, BC_2, P_1\text{ and } P_2 \). \( D \) represents additive variance effects, \( H \) is the dominance variance, \( E_w \) is the environmental variance, and \( F \) is the correlation between \( D \) and \( H \) over all loci calculated. Broad-sense and narrow-sense heritability was estimated according to Warner (1952):

\[
h_b^2 = \frac{[V_{F_2} - (V_{P_1} + V_{P_2} + 2V_{F_2})/4]}{V_{F_2}}, \\
h_n^2 = \frac{[2 V_{F_2} (V_{BC_1} + V_{BC_2})]}{V_{F_2}} \]

Results and discussion

Generations mean analysis

Significant differences were found among the generation means for carthamin and carthamidin contents. In both crosses of Mex.2-138×Wht- Esf and C111×Wht- Esf, the pigment concentrations content in the parental genotypes of C111 and Mex. 2-138 was more than that of Wht-Esf as the P2 parent in both crosses (Table 1). The F1 means for the evaluated crosses were between the means of the two parents. Also, the mean of F2 progenies was in the range of the parents mean for both traits. Back cross means of F1 to the superior parent \((P_1)\) showed a higher value for carthamidin and carthamin, as compared with the back cross mean of the inferior parent \((P_2)\). The classification of gene interactions depends on the magnitudes and signs of the estimates of dominance and dominance \(\times\) dominance effects, with many pairs of interacting genes (Mather and Jinks 1982). The sign associated with the estimates of \((a)\) and \((d)\) indicates the parent concentrating the highest number of genes for increasing the trait (Falconer and Mackay 1996). Therefore, the positive sign for \((a)\) in the evaluated indicates that the high pigments parent, C111 (carthamidin) and Mex.2-138 (carthamin) \((P_1)\), had the highest number of genes for increasing the pigments concentration.

The scaling tests for two different crosses are presented in Table 1. For both studied traits, none of the scales showed significance. The joint scaling is an effective combination of the whole set of scaling tests into one (Mather and Jinks 1982). This method is more convenient, informative and reliable than all other possible methods (2, 3, 4, 5 and 6...
parametric models) analyzed. The joint scaling test showed that the four terms (m, [a], [aa] and [ad]) were significant for carthamin and carthamidin in Mex.2-138× Wht- Esf and C111× Wht- Esf (Table 2). Chi square values were not significant in this study, indicating the data were fitted to the four parametric models including additive and epistatic ones. Hence, epistatic interactions including (additive × additive: i) and (additive × dominance: j) could be involved in the genetic control of carthamin and carthamidin. By considering that additive [a] and additive × additive [aa] had significant contributions in controlling carthamin and carthamidin, the gene interaction could be taken to be complementary (Mather and Jinks 1982). The additive × dominance [ad] was demonstrated at duplicate epistasis (Mather and Jinks 1982). The practical utilization of information obtained regarding epistasis in breeding could be a challenging issue that should be fully addressed by the scientists in the field of biometrics (Kearsey and Pooni 2004). The positive or negative form of additive × additive [aa] interaction showed the association or dispersion of alleles in parents, respectively. Therefore, the negative and significant values of [aa] in this study showed alleles dispersion in the parents of the two evaluated crosses for carthamin and carthamidin. Epistasis of the [aa] type could be used in the breeding programs with the additive component since it is fixable.
In relation to gene effects generation, the dominance gene effect could be assumed to be little because of ambidirectional dominance (Mather and Jinks 1982). We could conclude that this situation would be the case for the little dominance gene effects in carthamin and carthamidin inheritance.

**Generation variances analysis**

Genetic variances are the mean squares of each of locus effects not directly affected by gene dispersion and dominance (Khodambashi et al. 2012). Thus, data of generation variances could be used to complete genetic information (Nakhaei et al. 2014). Regarding generation variances, epistasis could only affect transgressive segregating generations variances (Mather and Jinks 1982).

Because of the presence of epistatic effects, the estimate of $V_A$ and $V_D$ components might not be unbiased. Variance estimation using the six generation values revealed that variation due to the additive genetic effect was predominant for carthamin, and variation due to the dominance genetic effect was predominant for carthamidin under study (Table 2). Additive genetic variance was predominant in the case of carthamidin and it was associated with homozygosity; hence, it was fixable in nature and selection for these characters could be very effective. Selection is the reliable breeding method employed for improving the safflower of carthamidin content. If the dominance is high, as much as carthamin inheritance, the selection has to be postponed to the later generation. Heterosis breeding is not desirable in the case of epistasis, but it would be possible to isolate the segregant, as well as that of $F_1$ in the subsequent filial generations (Singh and Pawar 2005). In this study, the estimates of $V_A$ and $V_D$ components of all traits were not free from bias because of the presence of epistatic gene effects. $V_A$ could be affected by the presence of [aa] and [ad], but, by considering the significant effect of the additive effects [a], the [aa] epistasis type could be used in the breeding programs with the additive component since it is fixable. The presence of [aa] often inflates the variance of $F_2$ and its subsequent generations (Khodambashi et al. 2012).

The generation variance and heritability estimates (broad–sense and narrow– sense) for traits are presented in Table 2. The heritability is not a property of a trait itself, but it is related to the population and environmental conditions (Falconer and Mackay 1996; Viana 2005). The high broad–sense heritability of both traits showed that the environment in which the plants were tested exerted a little effect on these traits, as compared to the genotype (Nakhaei et al. 2014). The studied traits, including carthamidin (48%) and carthamin (17%), had medium and low narrow-sense heritability, respectively, implying that most of the genetic variances were due to epistasis gene action. Heritability could be affected by epistasis (Khodambashi et al. 2012). The higher narrow sense heritability for carthamidin, rather than carthamin, indicated that selection breeding could be more effective in the improvement of carthamidin, not carthamin.

**Conclusion**

Safflower petal pigments including carthamin and carthamidin are expected to serve as valuable biochemical components for the improvement of medicinal and industrial properties of safflower. So, the information obtained on the genetics of safflower petal pigments could further aid plant breeders in selection of appropriate breeding methods for the coincident improvement of carthamin and carthamidin. Generation mean analysis of the data revealed additive and additive× additive epistatic effects involving the inheritance of two pigments of safflower
petals. Based on the obtained results, the breeding based selection could be effective for increasing these pigments contents in safflower petals. Alternatively, pursuing the preset materials with selfing and selection could result in advanced lines suitable for the public appeal, as well as medicinal purposes. Since the genetic improvement of medicinal, ornamental and industrial properties is one of the major goals of safflower breeding, the superior progenies in this study could be used in recombination breeding programs to accumulate suitable genes responsible for improving the mentioned traits.

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Yue S.J., Tang Y.P., Li S.J. and Duan J.A. 2013 Chemical and biological properties of quinochalcone C-glycosides from the florets of Carthamus tinctorius. Molecules, 18, 15220–15254.

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Table 1. Mean performance of six generation materials for the crosses of Mex.2-138× Wht- Esf and C111× Wht- Esf in safflower

<table>
<thead>
<tr>
<th>Generations</th>
<th>Mex.2-138× Wht- Esf</th>
<th>C111× Wht- Esf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obs. means± SE</td>
<td>Exp. means</td>
<td>Obs. means± SE</td>
</tr>
<tr>
<td>P1</td>
<td>5.98±0.57</td>
<td>5.98</td>
</tr>
<tr>
<td>P2</td>
<td>1.05±0.33</td>
<td>1.05</td>
</tr>
<tr>
<td>F1</td>
<td>5.85±0.67</td>
<td>5.87</td>
</tr>
<tr>
<td>F2</td>
<td>5.76±2.12</td>
<td>5.87</td>
</tr>
<tr>
<td>BC1</td>
<td>5.93±0.79</td>
<td>5.90</td>
</tr>
<tr>
<td>BC2</td>
<td>4.82±1.92</td>
<td>4.66</td>
</tr>
</tbody>
</table>

Scaling test

| | A | B | C | D |
| | 0.03±1.82 | 2.73±3.92 | 4.32±8.67 | 0.77±4.75 |
| | -1.29±3.52 | 12.66±17.05 | 17.79±25.79 | 3.21±15.43 |

¥: SE: Standard Errors.

Table 2. Estimation of genetic parameters and variance components in two different crosses of safflower.

<table>
<thead>
<tr>
<th>Crosses</th>
<th>Genetic parameters</th>
<th>Variance components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mex 2-138×Wht- Esf</td>
<td>4.87**±0.05</td>
<td>2.46**±0.033</td>
</tr>
<tr>
<td>C111× Wht- Esf</td>
<td>24.54**±0.27</td>
<td>12.42**±0.092</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variance components</th>
<th>( V_A )</th>
<th>( V_D )</th>
<th>( V_{AD} )</th>
<th>( V_E )</th>
<th>( b_r^2 )</th>
<th>( b_n^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mex 2-138×Wht- Esf</td>
<td>29.86</td>
<td>77</td>
<td>62.23</td>
<td>1.47</td>
<td>0.99</td>
<td>0.17</td>
</tr>
<tr>
<td>C111× Wht- Esf</td>
<td>9.52</td>
<td>6.74</td>
<td>3.06</td>
<td>0.33</td>
<td>0.98</td>
<td>0.48</td>
</tr>
</tbody>
</table>

* and ** significant at P<0.05 and P<0.01, respectively, using the least significant difference (LSD) test.
ns: not significant. ¥: means ± SE (Standard Errors).