

Research Article

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Testa and hilum colour associations with seed traits of a Greek field pea landrace

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Abstract

Pea landraces may hold genetic variation that can be exploitable in breeding/selecting new cultivars. In a small-seeded pea landrace, four phenotypes were distinguished according to testa (green, non-spotted and green, spotted) and hilum colour (white, black). The four phenotypes were tested for two growing seasons in the field for pod (seeds/pod) and seed traits (1000-seed weight, toughness, total phenols and tannins, testa colour, protein and carbon concentration, C/N ratio, bruchid infection). Significant differences were found for testa colour parameters, phenolic, tannin and carbon concentration and bruchid tolerance. The larger-seeded, spotted peas had darker testa and more phenols, while white-hilumed peas had lighter testa and more tannins. The spotted, black-hilumed phenotype, with the highest carbon concentration and C/N ratio was the most tolerant to bruchids. However, grouping the phenotypes, neither spotted nor black-hilumed ones showed to be more tolerant compared with their counterparts. Concluding, our results showed that phenotyping variation in seeds of a pea landrace revealed variation in seed traits, which could be exploitable. Since testa and hilum colour were associated with specific seed traits, they could, alone or in combination, be used as biomarkers of seed quality traits in pea. Testing of larger number of phenotypes is needed to solidify our findings.

Introduction

After being part of the daily diet of hunter-gatherers, peas (*Pisum sativum* L.) are being cultivated since antiquity participating in the diet of both modern humans and animals (Cousin, 1997; Smýkal *et al.*, 2012). The most widely cultivated pea varieties are *P. sativum* subsp. *sativum* var. *sativum* L. and *P. sativum* subsp. *sativum* var. *arvense* (L.) Poirlet, utilized for human consumption and livestock feed, respectively (Smýkal *et al.*, 2012; Weeden, 2018). Wild subspecies, like *P. sativum* subsp. *elatius* (Bieb.) Aschers. et Graebn. s.l., are considered as the basis for the domestication of cultivars (Weeden, 2007).

In Greece, split legume grains, mainly *Pisum* and *Lathyrus*, are used to cook fava puree (or fava), a local dish dating back to antiquity (Halstead and Glynis, 1989; Glynis and Halstead, 1993). In insular Greece (e.g. islands Schinoussa, Amorgos, Skyros), small-seeded landraces of *P. sativum* subsp. *sativum* are used for fava; these landraces belong to *P. sativum* subsp. *sativum*, but are closely related to subsp. *elatius*, the wild subspecies (Stavridou *et al.*, 2020).

Pisum sativum is a species with inherently high diversity in agronomic and seed traits (Annicchiarico *et al.*, 2017; Solberg *et al.*, 2017; Gali *et al.*, 2019), an indispensable prerequisite for the genetic improvement of the crop (Swarup *et al.*, 2021). In this line, landraces, which are genetically diverse, locally adapted, lacking of formal crop improvement populations (Camacho Villa *et al.*, 2005), may host genetic diversity potentially exploitable for crop improvement in the challenging future conditions (Dwivedi *et al.*, 2016; Preston *et al.*, 2019).

Pea genotypes show high variability for grain nutritive components like protein or starch (Annicchiarico *et al.*, 2017; Solberg *et al.*, 2017; Tao *et al.*, 2017; Mohammed *et al.*, 2018), but also for their phenolic content and thus antioxidant activity (Devi *et al.*, 2019; Zhao *et al.*, 2020). It has been found that phenolic content is associated with seed coat (testa) colour with dark-seeded peas being enriched in phenolics (Xu *et al.*, 2007; Jha *et al.*, 2019) and it was suggested that they could be used as a functional food (Zhao *et al.*, 2020). Apart from testa colour, hilum colour has been found to associate with seed traits like phenolic content (Zhao *et al.*, 2020). If associated with certain seed traits, both testa and hilum colour could be used as handy and friendly biomarkers for phenotypic breeding/selection efforts (Chukwumah *et al.*, 2009; Reynolds *et al.*, 2020).

Seed phenolics could act as a chemical defence against pea weevil (bruchid, *Bruchus pisorum* L.), the major insect pest of dry peas, which claims a very high (>60%) yield toll yearly (Teshome *et al.*, 2015). Selecting and growing tolerant genotypes is a low-cost, effective and sustainable way to cope with bruchids (Mishra *et al.*, 2018). Traits like seed size, testa colour



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and seed toughness (ST) have been reported to associate with tolerance to bruchids. Small seeds, offering less food to developing larvae, are also tougher and thus more tolerant to bruchids (Sadakiyo and Ishihara, 2012; Fricke and Wright, 2016; Tsialtas *et al.*, 2018). Also, dark-seeded peas were found to be less susceptible, possibly due to the higher phenolic contents (Teshome *et al.*, 2015; Zhao *et al.*, 2020).

The present study aimed to identify putative associations between testa and hilum colour with seed traits (seeds/pod, protein concentration (PC), 1000-seed weight (TSW), phenolic contents, toughness, tolerance to bruchids) in four phenotypes of a field pea landrace in order colour to be used as phenotypic biomarker in selection schemes.

Materials and methods

Genetic material and experimental set up

Four phenotypic accessions were selected out of the seeds of 'Katsouni' pea landrace (*P. sativum* L. subsp. *sativum*; Tsialtas and Tan, 2017) from Schinoussa island (36°52' N, 25°31' E). The selection was based on testa (green or green with black spots) and hilum colour (white or black) and the four phenotypes were encoded as green testa and white hilum (T_GH_W), green testa and black hilum (T_GH_B), spotted testa and white hilum (T_SH_W) and spotted testa and black hilum (T_SH_B).

The experiment took place in the farm of Aristotle University of Thessaloniki (40°32'1 N, 22°59'3 E, 0 m a.s.l.) on a typical xerorthent loam, under rain-fed conditions, during 2016–2017 (hereafter 2017) and 2017–2018 (hereafter 2018) growing seasons. The growing season of 2018 (December to May) was wetter and warmer than the 2017 growing season (255 versus 179 mm and 13.5 versus 10.5°C).

The arrangement of the four phenotypes followed the randomized complete block (RCB) design with three replications. Each plot (1 m² area) consisted of four, 1 m long rows, at 0.25 m separation in accordance to our previous works (Tsialtas *et al.*, 2018, 2020; Boulata *et al.*, 2022). The seeding, at a rate of 40 seeds/m² (10 seeds per row), was conducted by hand on 3 December 2016 and 7 December 2017. The blocks were separated by a 1.5 m buffer zone. No fertilization or irrigation was supplied and the weeds were removed by hand, when necessary.

Assessments on pods and seeds

At full seed maturity (BBCH 89; Lancashire *et al.*, 1991), all the pods were collected from the two internal rows of each plot and kept in paper bags. Fifteen randomly selected pods per plot were used to measure the number of seeds/pod (SpP) and the mean value per plot was calculated. Then, all the pods per plot were threshed by hand and seeds kept in paper bags at room temperature and darkness.

Hundred seeds per plot were randomly selected and placed in plastic boxes (150 ml) and kept at room temperature (25/20°C day/night temperature) for over three months until exits of the adult bruchids consummated. The damage caused by bruchids and its endoparasitoid wasp (*Triaspis thoracica* Curtis, 1860, Hymenoptera: Braconidae) on the seeds was estimated macroscopically by examining all the seeds per box as described by Tsialtas *et al.* (2018). The bruchid infestation level (BI) was calculated as the sum of bruchid-damaged (BD) and parasitoid-damaged (PD) seeds and expressed in percentage (%).

Seed coat colour was assessed using a CR-400/410 chroma meter (Konica Minolta, Kyoto, Japan). Uninfected seeds of each plot were placed in a cylindrical petri dish, 39 mm internal diameter, firmly fixed to instrument's glass light projection tube (CR-A33a). Chroma meter measurements were based on the International Commission of Illumination (CIE) colour solid scale (L^* , a^* , b^*). Specifically, L^* represents lightness (from black = 0 to white = 100), a^* is for greenness and redness (red = positive value and green = negative value) and b^* corresponds to blueness and yellowness (yellow = positive value and blue = negative value). Details on the testa colour assessment are given by Tsialtas *et al.* (2018).

A sample of 100 intact seeds per plot was dried at 75°C till constant weight. Dry weights were estimated and TSW was calculated by multiplying the dry weights by 10.

From the samples used for TSW calculations, 10 randomly selected seeds per sample were used to assess ST, the essential energy (J/g) for seed breaking by compression test. For the ST measurements, a texture analyser (TA-XT2i, Stable Micro Systems, Godalming, Surrey, UK) was used and the procedure followed Tsialtas *et al.* (2018). The reported value for each plot represented the average of the 10 measurements. The lower the fracture energy, the softer the seeds were.

All the 100 dried seeds used for TSW calculations were ground to fine powder using an ultra-centrifugal mill ZM-1000 (Retsch GmbH, Haan, Germany) equipped with a 0.5 mm sieve. Per plot, a subsample of ca. 1.0 mg was weighed in order to determine both seed carbon (C) and nitrogen (N) concentrations on an elemental analyser (EuroEA 300, EuroVector SpA, Milan, Italy) and the concentrations were expressed in per cent (%). Seed PC was estimated as the product %N × 6.25 and the carbon to nitrogen ratio (C/N) was also calculated.

To measure total phenols (TPhe) and tannins (TTan), ca. 0.2 g of grounded seed material per plot was extracted with 4 ml aqueous acetone (70% v/v) using ultrasonic treatment for 15 min and then, the samples were centrifuged at 2200 rpm for 10 min. After the collection of supernatants, the pellets were treated once more applying the abovementioned procedure. Folin–Ciocalteu reagent was used for the detection of total phenols and tannins. The evaluation of total phenols in the samples is a result of their absorption at 725 nm and total tannins were calculated after subtraction of non-tannin phenolics from total phenolics. Results were expressed in mg of gallic acid equivalents (mg GAE) per g sample (Tsialtas *et al.*, 2018).

Statistical analysis

Data analysis was performed based on the over-year analysis of variance as RCB design with pea phenotypes, testa colour and hilum colour as main factors and three replications. Data were analysed using M-STAT statistical package (M-STAT C, version 1.41, Crop and Soil Science Department, Michigan State University, USA). The least significant difference (LSD) test was used for means comparisons, considering $P < 0.05$ as significance level.

The Microsoft Excel software (v. 2010) was used to construct graphs and estimate correlation coefficients (r) between the traits for which statistically significant differences were found among phenotypes and their interaction with growing seasons. In order to avoid spurious self-correlations, coefficients were not calculated for interconnected traits, e.g. BD-BI, PD-BI, TPhe-TTan.

Results

The main factors (phenotypes, testa colour, hilum colour and growing seasons) explained the largest part of the variation of the determined traits and thus, the mean comparisons are presented for these factors.

Percentages of damaged seeds, morphological, mechanical and chemical traits

The four phenotypes differed in the percentage of bruchid infected seeds (BI) and seed phenolic and tannin concentration (Table 1). The T_SH_W (spotted testa, white hilum) showed to be more susceptible to bruchids (62.5%) compared to the rest three phenotypes (44.50–54.50%). It also had the highest TTan (4.51 mg GAE/g) and along with the T_SH_B (spotted testa, black hilum) showed the highest TPhe (7.46–7.49 mg GAE/g).

On one hand, non-spotted phenotypes (T_GH_W and T_GH_B combined) had smaller seeds (TSW: 71.53 g versus 77.60 g) compared to spotted phenotypes (T_SH_B and T_SH_W combined), but lower TPhe (6.55 versus 7.47 mg GAE/g). On the other hand, the phenotypes with black hilum (T_GH_B and T_SH_B combined) had lower TTan (3.77 versus 4.54 mg GAE/g) compared to the phenotypes with white hilum (T_GH_W and T_SH_W combined).

Growing seasons had a significant effect on all the determined traits. In 2017 growing season, bruchid and endoparasitoid infections (BD, PD, BI), TPhe and TTan were higher, but SpP, seed weight (TSW) and ST were lower compared to 2018 (Table 1).

Testa colour and nutrient concentrations

The four phenotypes differed significantly in testa colour and seed nutrient parameters with the exception of seed PC (Table 2). *L*^{*} (lightness) was highest in T_GH_W (36.11), moderate in T_GH_B

and T_SH_W (32.00 and 32.31, respectively) and lowest in T_SH_B (30.53). The T_SH_B phenotype had the highest *a*^{*} (−0.27), but the lowest *b*^{*} value (12.31), while the adverse was found for T_GH_W. The highest carbon concentration was measured in T_SH_B (44.58%), while the lowest in T_SH_W (42.11%), which also had the lowest C/N ratio (9.46).

No differences were found between non-spotted and spotted phenotypes for seed nutrient composition (Table 2). The non-spotted phenotypes had higher *L*^{*} (34.27 versus 31.42) and *b*^{*} values (16.62 versus 13.16), but lower *a*^{*} (−2.44 versus −0.80) compared to spotted ones.

Hilum colour had no effect on PC and C/N ratio (Table 2). On the other hand, phenotypes with white hilum had higher *L*^{*} (34.21 versus 31.49) and *b*^{*} values (16.16 versus 13.62), but lower *a*^{*} (−2.28 versus −0.95) and carbon concentration (42.51 versus 43.90%).

With the exception of *a*^{*} and PC, growing seasons affected the testa colour and seed nutrient traits, all of which were higher in 2018 growing season (Table 2).

Relationships between the traits differed significantly among phenotypes

Among the four phenotypes, percentage of bruchid infection (BI) was negatively correlated with seed carbon concentration ($r = -0.98$, $P < 0.05$, $n = 4$) and C/N ratio ($r = -0.98$, $P < 0.05$, $n = 4$) (Fig. 1).

Testa colour parameters (*L*^{*}, *a*^{*}, *b*^{*}) were significantly inter-correlated positively (*L*^{*}–*b*^{*}: $r = -0.98$, $P < 0.05$, $n = 4$) or negatively (*L*^{*}–*a*^{*}: $r = -0.98$, $P < 0.05$, $n = 4$ and *a*^{*}–*b*^{*}: $r = -1.00$, $P < 0.05$, $n = 4$) (Fig. 2).

For the phenotypes × growing seasons interaction, a negative correlation was found between total tannin concentration and seed C/N ratio (Fig. 3).

Table 1. Mean comparisons of the number of seeds per pod (SpP), percentages of damaged seeds [bruchid-damaged (BD), parasitoid-damaged seeds (PD) and percentage of bruchid infected seeds (BI)], 1000-seed weight (TSW), seed toughness (ST), total phenolic compounds (TPhe) and total tannins (TTan)

Phenotypes	SpP	BD %	PD %	BI %	TSW g	ST J/g	TPhe mg GAE/g	TTan mg GAE/g
T _G H _B	6.77a	26.83a	27.67a	54.50ab	71.47a	2.89a	6.71ab	3.60b
T _G H _W	6.92a	26.83a	27.00a	53.83ab	71.60a	2.88a	6.39b	3.97b
T _S H _B	6.67a	19.17a	25.33a	44.50b	78.53a	2.61a	7.46a	3.94b
T _S H _W	6.91a	31.50a	31.00a	62.50a	76.68a	2.61a	7.49a	4.51a
CV (%)	4.63	30.32	37.72	17.05	9.07	14.03	9.94	10.41
Testa colour								
Non-spotted	6.85a	26.8a	27.3a	54.1a	71.53b	2.88a	6.55b	3.78a
Spotted	6.79a	25.3a	28.2a	53.5a	77.60a	2.61a	7.47a	4.22a
CV (%)	5.05	28.61	41.64	22.4	6.79	12.5	13.52	14.41
Hilum colour								
Black	6.72a	23.0a	26.5a	49.5a	75.00a	2.75a	7.08a	3.77b
White	6.92a	29.2a	29.0a	58.2a	74.14a	2.74a	6.94a	4.54a
CV (%)	3.93	35.11	38.56	21.1	8.73	13.54	11.57	12.62
Growing seasons								
2017	6.63b	31.2a	41.7a	72.8a	69.19b	2.58b	8.08a	4.43a
2018	7.01a	21.0b	13.8b	34.8b	79.94a	2.91a	5.95b	3.58b

For the same column and factor (phenotypes, testa colour, hilum colour, growing seasons), means followed by the same letter did not differ significantly using LSD at $P < 0.05$. Where CV is coefficient of variation.

Table 2. Mean comparisons of seed coat colour parameters (L^* , a^* , b^*), protein concentration (PC), seed carbon concentration (C) and carbon to nitrogen ratio (C/N)

Phenotypes	L^*	a^*	b^*	PC %	C %	C/N
T _G H _B	32.00b	-1.64b	14.93b	26.21a	43.23ab	10.34a
T _G H _W	36.11a	-3.24c	18.30a	25.96a	42.91ab	10.33a
T _S H _B	30.53c	-0.27a	12.31c	25.59a	44.58a	10.92a
T _S H _W	32.31b	-1.33b	14.01b	28.11a	42.11b	9.46b
CV (%)	3.99	45.67	5.65	7.74	3.26	5.29
Testa colour						
Non-spotted	34.27a	-2.44b	16.62a	26.85a	43.34a	10.19a
Spotted	31.42b	-0.80a	13.16b	26.09a	43.07a	10.34a
CV (%)	3.55	42.17	5.22	8.97	3.52	8.59
Hilum colour						
Black	31.49b	-0.95a	13.62b	25.09a	43.90a	10.63a
White	34.21a	-2.28b	16.16a	27.03a	42.51b	9.9a
CV (%)	4.34	50.16	7.36	9.21	3.31	8.79
Growing seasons						
2017	30.47b	-1.81a	12.73b	26.30a	40.95b	9.82b
2018	35.22a	-1.43a	17.04a	26.63a	45.46a	10.71a

For the same column and factor (phenotypes, testa colour, hilum colour, growing seasons), means followed by the same letter did not differ significantly using LSD at $P < 0.05$. Where CV is coefficient of variation.

Discussion

Pea (*P. sativum* L.) landraces hold considerable genetic diversity, which could be exploitable via breeding/selection (Keneni *et al.*, 2005; Gixhari *et al.*, 2014; Hagenblad *et al.*, 2014). This was confirmed in the present study by the differentiation of the four phenotypes in seed chemical (total phenols, total tannins, C concentration and C/N ratio) and testa colour traits, but more interesting in bruchid infection (BI) susceptibility.

Testa colour parameters (L^* , a^* , b^*) showed significant inter-correlations and in accordance to Zhao *et al.* (2020), a positive correlation between L^* (lightness) and b^* (yellowness) was evident. These researchers reported negative correlations of seed phenolic concentrations with L^* and b^* , which were not confirmed for the four phenotypes in our work. The findings of Zhao *et al.* (2020) were in agreement with previous reports that pea seeds with darker testa had more phenolics and higher antioxidant capacity (Troszyńska and Ciska, 2002; Xu *et al.*, 2007; Marles *et al.*, 2013; Jha *et al.*, 2019). However, when the four phenotypes grouped according to testa and hilum colour, our findings were consistent with Zhao *et al.* (2020); the larger, spotted seeds contained more phenolics and had lower L^* and b^* values. On the other hand, the white-hilumed seeds contained more tannins, had higher L^* and b^* values and higher C concentrations.

Seed weevil is the major insect pest of dry peas with very high levels of infection, especially in cream-coloured peas where infection percentages can reach 98% (Teshome *et al.*, 2015). The two spotted phenotypes had the lowest (44.50%) and the highest levels (66.50%) of BI, with white-hilumed phenotype (T_SH_W) to be the most susceptible. This finding comes to confirm previous reports for genotypic differences in bruchid tolerance in grain legumes like peas (Teshome *et al.*, 2015; Nikolova, 2016), vetches (Tsialtas *et al.*, 2018; Boulata *et al.*, 2022), faba beans (Seidenglanz and Huňady, 2016; Dell'Aglio and Tayeh, 2023)

and red pea (*Lathyrus cicera* L.) (Tsialtas *et al.*, 2020). Finding easily assessable phenotypic traits like testa and hilum colour, related to bruchid tolerance, could be a useful and handy tool

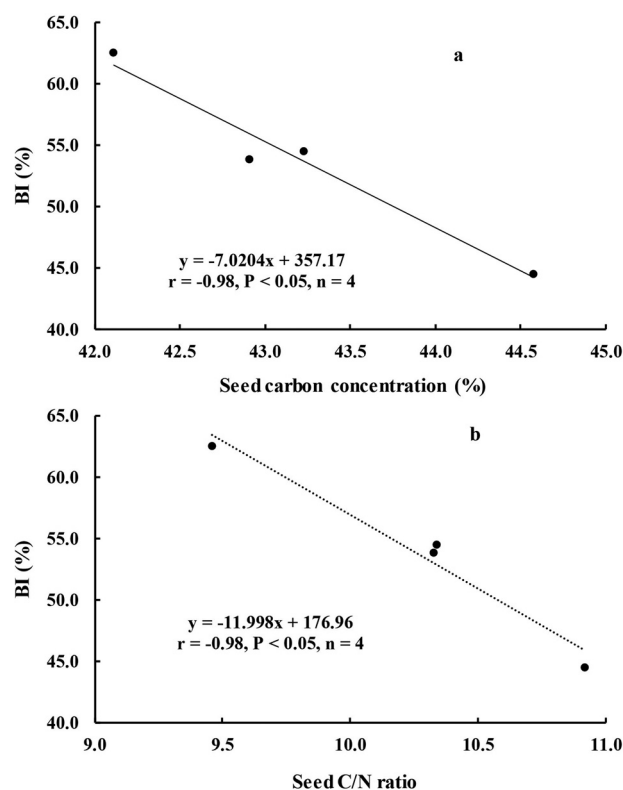


Figure 1. Correlations between percentage (%) of bruchid infection (BI) with seed carbon concentration (a) and C/N ratio (b).

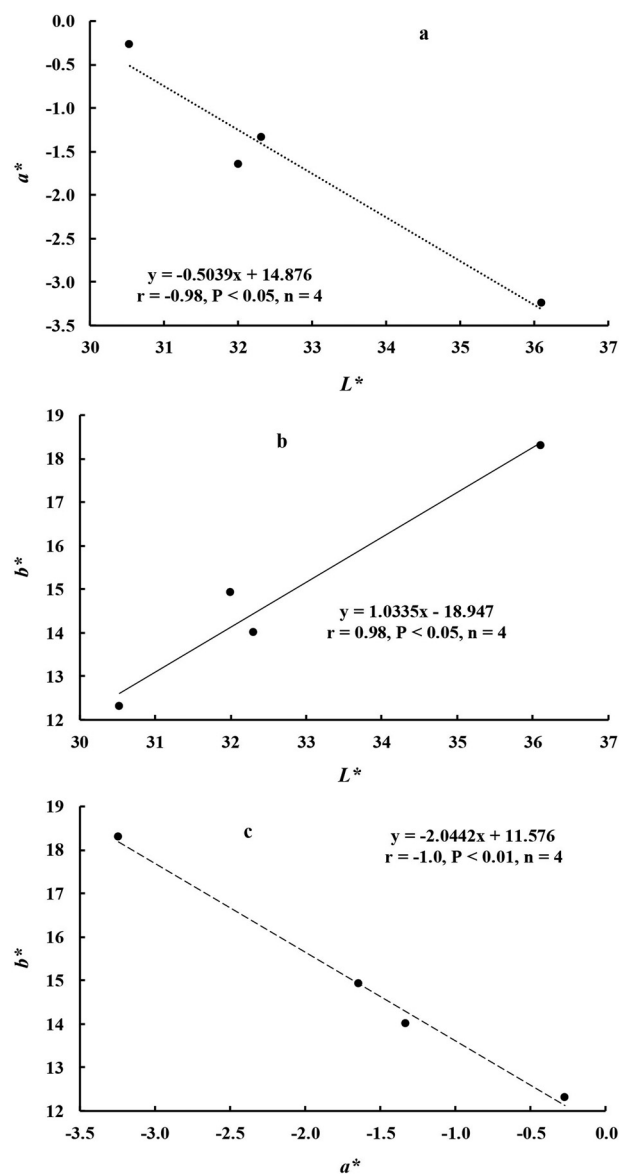


Figure 2. Inter-correlations between testa colour parameters (L^* , a^* , b^*).

in respective selection/breeding efforts. However, in our case, such generalization cannot be done since when the data combined over testa or hilum colour, no difference was evident.

Previously, tolerance to bruchids has been associated with small seed size (Fricke and Wright, 2016; Tsialtas *et al.*, 2018), low seed N and P concentrations (Nikolova, 2016), high ST (Tsialtas *et al.*, 2018) and toxic effects of high phenolic or metal (e.g. Fe) concentrations (Barbehenn and Constable, 2011; Tsialtas *et al.*, 2020). In our case, the tolerance of the four phenotypes to bruchids was related with higher seed C concentration and C/N ratio meaning that seeds with relatively higher N concentration (lower C/N) were more preferable by bruchids, which was in line with the findings of Nikolova (2016). The inverse relationship between seed C/N ratio and total tannins for the phenotypes \times growing seasons interaction inferred that seeds with higher tannin concentration were more susceptible to bruchids, which contradicts the inhibitory role of tannins to feeding insects (Barbehenn and Constable, 2011). However, the synthesis of the costly tannins as a chemical defence means is not always an

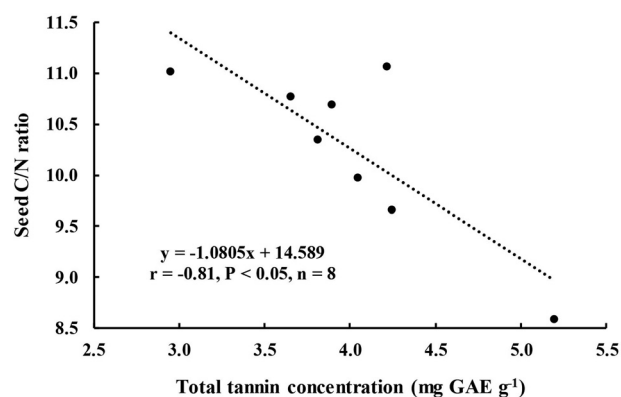


Figure 3. Total tannin concentration in seeds was correlated with C/N ratio for the phenotypes \times growing seasons interaction.

option for the plants, but it depends on abiotic factors (e.g. soil nutrient and water availability), the growth type of the plant (fast- or slow-growing) and is combined with other chemical defensive substances (Seigler, 2002).

Conclusions

The pea landrace ‘Katsouni’ holds phenotypic variation in testa and hilum colour, which was associated with seed trait variation. Spotted seeds with darker testa had more phenolics, while white-eyed seeds contained more tannins. Seeds with spotted testa and black hilum were less susceptible to bruchids.

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Competing interest. None.

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